

## DETERMINATION OF THYROID-BINDING INHIBITING IMMUNOGLOBULINS USING UNEXTRACTED SERUM

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### Summary

A simplified assay for the detection of Thyroid-binding Inhibiting Immunoglobulin (TBII) using unextracted serum was set up. A total of 176 patients with Graves' disease were tested for the presence of TBII in their serum. Using the +3SD limit for normal controls as the cut off value for a positive result, 124 patients (70.5%) were positive for TBII. A further 18 patients (10.2%) who had TBII values between +2SD and +3SD limits were considered borderline positives. The intra- and inter-assay variability, determined by replicate analysis of samples from patients with Graves' disease were both good at 5.8% and 5.6% respectively. Assay results obtained were comparable to that of standard procedure using extracted gamma-globulins as samples. Performance characteristics of the assay were acceptable.

Keywords: Thyroid-binding Inhibiting Immunoglobulin, thyroid, Graves' disease.

### INTRODUCTION

The thyroid stimulator present in the serum of patients with Graves' disease was first described in 1956 by Adam and Purves.<sup>1</sup> This abnormal stimulator differed from thyroid stimulating hormone (TSH) in its prolonged duration of action, hence the designation long-acting thyroid stimulator (LATS). Initial assays for this were based on its biological activity.<sup>2</sup> This was then superseded by radioreceptor assays which depended on the ability of the thyroid stimulator to bind to thyroid extracts, thereby inhibiting the binding of <sup>125</sup>I-labelled thyroid stimulating hormone (TSH).<sup>3-5</sup> Immunoprecipitation assays using solubilised TSH-receptors have more recently been described.<sup>6-8</sup>

Current assays for the detection of abnormal thyroid stimulators in patients with Graves' disease are based on the TSH receptor assay. Among the problems associated with this assay is the interference, by normal serum proteins, of binding of the <sup>125</sup>I-labelled TSH.<sup>8-11</sup> As a result, most assays use extracted gamma-globulins instead of serum samples. However, it has been demonstrated that gamma-globulins in themselves cause appreciable suppression of binding at a concentration of 5g/l, and it was suggested that the observed interference by serum proteins is artefactual.<sup>12</sup> If this is the case, the problem can be minimised by optimization of assay conditions. The objectives of the present

study were (i) to develop a simplified in-house assay for the detection of thyroid-binding inhibiting activity in patients with Graves' disease based on an optimised TSH-receptor assay<sup>13</sup> and (ii) to determine whether unextracted serum is suitable as the sample for analysis.

### MATERIALS AND METHODS

#### Specimens

Serum specimens were collected from unselected patients with clinical features of Graves' disease and biochemical evidence of hyperthyroidism including newly diagnosed patients, known cases with relapse and patients on treatment. Samples were stored in suitable aliquots at -70°C until analysis.

Serum specimens were collected from normal blood donors for use as negative controls. A pooled normal serum was prepared from these specimens for use as a control in each assay.

#### Reagents

**Thyroid membranes:** Fresh thyroid specimens were obtained at thyroidectomies from patients with Graves' disease and non-toxic goitres. Crude thyroid membranes were prepared by homogenization and differential centrifugation. Thyroid membranes were stored in suitable aliquots at -70°C until use. Membranes were tested for tracer binding capacity using a

bovine TSH (bTSH) receptor assay to allow for selection of membrane preparations with adequate binding for the TBII assay.

<sup>125</sup>I-bovine TSH was prepared by labelling highly-purified bTSH (a gift from Dr. Pierce, University College of Los Angeles School of Medicine, California), using the Bolton-Hunter conjugation method (Specific Activity : 1–2 Bq/pg.). The labelled bTSH was initially purified by chromatography through two sephadex columns and then receptor-purified prior to use in the TBII assay. The receptor-purified label was kept in low ionic buffer (10 mM NaCl, 10 mM Tris/HCl, 1 g/l BSA, pH 7.5) and stored frozen at –70°C.

**Procedure**

The procedure was based on an optimised TSH receptor assay described by J.C. Kermode.<sup>8</sup> Briefly, it involved duplicate incubation of 100 ul of neat, unextracted serum samples with about 40 pg of receptor-purified <sup>125</sup>I bTSH and 5 mg -equivalent of thyroid membrane (i.e. that amount of thyroid membrane obtained from 5 mg of thyroid tissue). Each of the two latter reagents were contained in low ionic strength buffer (10 mM NaCl, 10 mM Tris/HCl, 1 g/l BSA, pH 7.5) to correct for the high salt concentration of the serum samples. Previous studies have demonstrated that the optimal ionic strength for the present assay procedure was between 40–60 mM (unpublished observations). Simultaneously, control tests were set up using (i) a pooled normal serum, (ii) a TBII positive sample, (iii) human TSH standards at 0.5 mu/ml and at 100 mu/ml (the latter to determine non-specific binding) and (iv) normal saline. A series of serum samples from normal blood donors were also tested to assess the variation of binding of <sup>125</sup>I-bTSH in the presence of different normal sera.

The mixtures were incubated at 37°C for 2 hours, after which the reaction was stopped by addition of 1.0 ml of ice-cold buffer (40 mM NaCl, 10 mM Tris/HCl, 1 g/l BSA, pH 7.5) and centrifugation at 25000G for 15 minutes at 4°C. The pellets were counted for <sup>125</sup>I

**RESULTS**

Results were expressed as the Thyroid-binding Inhibiting Immunoglobulin (TBII) Index, which is a measure of the percentage suppression of the specific binding of <sup>125</sup>I bTSH:<sup>14,15</sup>

$$TBII \text{ Index} = 100 (B_N - B_S) / B_N$$

$B_N$  = specific binding for <sup>125</sup>I bTSH in the presence of normal serum.

$B_S$  = specific binding for <sup>125</sup>I bTSH in the presence of test sample.

The average binding of <sup>125</sup>I bTSH in the presence of isotonic saline was about 20%, while that in the presence of normal serum was 12.5%. The percent reduction of binding in the presence of pooled serum was 36.6 ± 7.8%. Non-specific binding (NSB) generally varied between 1% to 2%.

A total of 105 normal sera were tested. The coefficient of variation (CV) of binding of these specimens in different assays varied between 4.0% and 10.5%. The combined estimate (weighted root-mean square value) of the CV was 6.6%. The intra-assay and inter-assay variability were determined by replicate analysis of samples from patients with Graves' disease. The within-assay variability was 5.8% (mean TBII = 52.5%, SD = 3.2%).

Unextracted serum obtained from 176 patients with Graves' disease were tested for presence of TBII. The TBII index of these patients ranged from –3% to 92%. The +3SD limit for normal controls was taken as the cut-off value for a positive result. Values falling between +2SD and +3SD limits were considered borderline positive results. Based on these limits, 124 patients (70.5%) were positive for TBII, 18 patients (10.2%) were borderline positive and the remaining 34 cases (19.3%) were negative (Fig. 1).

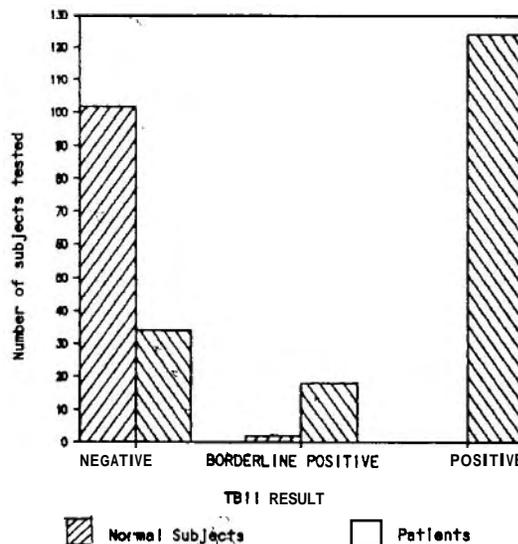


FIG. 1: TBII results of patients with Graves' disease compared with normal subjects.

A similar assay using polyethyleneglycol (PEG) extracted immunoglobulins instead of neat serum samples was performed to check the present assay procedure. Results obtained were comparable to those using serum samples assayed in parallel. The average decrease in binding in the presence of normal immunoglobulin (Ig) extract was 39%, slightly higher than that obtained with normal serum (36%). The pooled normal Ig specimen gave similar suppression of 38%. The mean TBII index of the normal Ig specimens was 0.3% and the SD was 6.36% while that of the normal sera were 0.2% and 4.04% respectively. Results of 19 specimens assayed in parallel using Ig extracts and neat serum are shown in Fig. 2.

## DISCUSSION

Using an optimised bTSH receptor assay, a simplified in-house assay for the detection of thyrotropin-binding inhibitory activity in serum was developed. The assay procedure is essentially similar to most assays described in the literature. However, we have modified the assay to allow for the use of unextracted serum samples instead of extracted gamma globulins. All membrane preparations used were checked for quality with regard to TSH-binding activity prior to use. Five mg equivalent of thyroid membranes were used instead of the usual 15–20 mg equivalent to increase sensitivity. Bovine TSH tracer was iodinated to a low

specific activity of between 1–2 Bq/pg to give approximately one atom of iodide per molecule of hormone. The tracer was first purified by chromatography and then repurified by binding to and subsequent elution from human thyroid membranes. Receptor-purified tracer was used within one week of preparation. The assay procedure involved simultaneous incubation of test or control serum with tracer and thyroid membrane, the latter two components being in low ionic strength buffer to correct for the high sodium concentration in serum. A pooled normal serum, a human TSH standard and a known TBII positive sample were included in each assay to check for comparability between assays.

15 separate assays were performed using 3 different thyroid membrane-preparations. The binding of  $^{125}\text{I}$  bTSH in the presence of pooled normal sera averaged 12.5%, while that in the presence of isotonic saline averaged 19.8%, giving an average percentage reduction in tracer binding of  $36.6 \pm 7.8\%$ . The coefficient of variation of binding in the presence of 105 different-normal sera varied between 4.0% to 10.5% in each of these assays with a combined estimate of 6.6%. Taking this value as representative of all assays, samples with a TBII index more than 19.8% were regarded as positive and those between 13.2% and 19.8% borderline.

The observed interference of binding of  $^{125}\text{I}$  bTSH by normal serum was not greater than that observed using PEG precipitated gamma-globulins, which suggests that use of a globulin extract offers no advantage over the use of neat serum. The degree of interference by normal sera was lower than that reported with other procedures.<sup>9,11,15</sup> Further, the spread of tracer binding percentages in the presence of the different normal sera (CV = 6.6%) was narrower than that reported using globulin extracts.<sup>6,17</sup>

Using this procedure, we tested serum samples obtained from 176 unselected patients who were diagnosed clinically and biochemically as thyrotoxic. Overall, the TBII indices of these subjects ranged from -3% to 92%, of whom 124 cases were considered positive and another 18 were borderline positive for TBII.

Using the +3SD limit as the cut-off, about 71% of the subjects were positive for TBII, a level lower than that reported by some other investigators.<sup>6,12</sup> This could be accounted

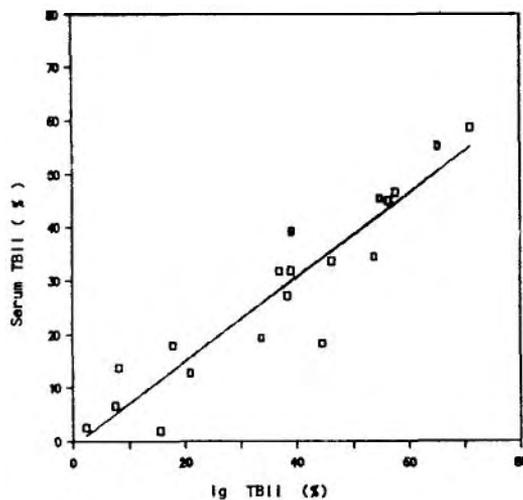


FIG. 2: Serum TBII results compared with Ig TBII results ( $y = 0.78 X - 0.76$ ,  $r = 0.93$ )

for in part by the selection of cases for study. If selection was limited only to new cases, and did not include previously or currently treated patients, the frequency of positive results may be expected to be higher. Another possibility is inadequate sensitivity of the assay. It was noted that the receptor-purified label kept in low ionic strength buffer tended to deteriorate on storage, with decreasing percentage binding and increasing non-specific binding. In contrast, label stored in higher ionic strength (40 mM NaCl, 10 mM Tris-HCl, 1 g/l BSA, pH 7.5 buffer) was observed to exhibit better binding using the same membrane preparations, and was more stable on storage. Further, we have experienced problems with the quality of the Bolton-Hunter reagent received. This was believed to be due to problems of transportation, often resulting in the reagent being delayed, during which period it could conceivably have been kept under conditions that cause deterioration of this relatively unstable reagent.

The coefficients of intra- and inter-assay variation at 53% and 58% inhibition of labelled TSH binding were acceptable, being 5.8% and 5.6% respectively. This may possibly be improved further by the use of thyroid membrane preparations obtained from a single patient and a more stable  $^{125}\text{I}$  bTSH preparation. The other receptor assay which showed a relative lack of interference of binding of labelled TSH by normal serum was that using detergent solubilised receptors.<sup>6</sup> Even with this procedure, the use of 100  $\mu\text{l}$  neat serum from normal subjects produced inhibition of binding of  $10.7 \pm 9.2\%$  (mean  $\pm$  SD inhibition of TSH binding). Although we experienced an average inhibition of tracer binding of about 36% in the presence of 100  $\mu\text{l}$  of normal sera (compared with normal saline), this was not appreciably greater than that found with the use of extracted globulins. Further, the coefficient of variation for the binding of  $^{125}\text{I}$  b-TSH in the presence of different individual normal sera was sufficiently low and comparable to that reported using the solubilised membrane assay. Nevertheless, further improvements to the assay is necessary, in terms of better standardization and improvement of assay sensitivity. Use of a single membrane preparation or membrane preparations with similar binding characteristics and sensitivity to TBII might overcome the variation in binding related to different membrane preparations. Another possibility is the use of pooled membrane preparations. Improvement of the tracer can

be achieved by storage in higher ionic strength buffer, and rechromatographing into the appropriate buffer just prior to use. To overcome the problem of supply of the Bolton-Hunter reagent, the use of alternative markers including an enzyme label can be considered.

The present study has demonstrated that it is possible to measure serum TBII in patients with Grave's disease using unextracted serum specimens. Results obtained are comparable to those using extracted gamma-globulins. The performance characteristics of the assay are acceptable. However, it appears that further optimization can be achieved by improvement of the quality of the tracer and by standardization of membrane preparations.

#### REFERENCES

1. Adams DD, Purves HD. Abnormal responses in the assay of thyrotrophin. Proceedings of the University of Otago Medical School 1956; 34: 11-2.
2. McKenzie JM. Delayed thyroid response to serum from thyrotoxic patients. Endocrinology 1958; 62: 865-8.
3. Mehdi SQ, Nussey SS, Gibbons CP, el Kabir DJ. Binding of thyroid stimulators to human thyroid membranes. Biochem Soc Trans 1973; 1: 1005-6.
4. Manley SW, Bourke JR, Hawker RW. The thyrotrophin receptor in guinea-pig thyroid homogenate: interaction with the long-acting thyroid stimulator. J Endocrinol 1974; 61: 437-45.
5. Smith BR, Hall R. Thyroid stimulating Immunoglobulins in Graves' disease. Lancet 1974; ii: 427-31.
6. Shewing G, Rees-Smith B. An improved radioreceptor assay for TSH Receptor antibodies. Clin Endocrinol 1982; 17: 409-17.
7. Kotulla P, Schleusener H. Solubilised TSH receptor: its usefulness for the radiolig and receptor assay for TSH and TSH-displacing antibody. J Endocrinol 1981; 4: 155-60.
8. Rees Smith B, Pyle GA, Petersen VB, Hall R. Interaction of thyroid-stimulating antibodies with the human thyrotrophin receptor. J Endocrinol 1977; 75: 401-7.
9. Doctor R, Bos G, Visser TJ, Henneman G. Thyroid Binding Inhibiting Immunoglobulin in Graves' disease before, during and after antithyroid therapy, and its relation to long-acting thyroid stimulator. Clin Endocrinol 1980; 12: 143-53.

10. Davies TF. Autoantibodies to the human thyrotrophin receptor are not species specific. *J Clin Endocrinol Metab* 1981; 52: 426-X).
11. Brown RS, Kertiles LP, Reichlin S. Partial purification and characterisation of thyrotrophin binding inhibiting immunoglobulin from normal human plasma. *J Clin Endocrinol Metab* 1983; 56: 156-63.
12. Kermode JC. An investigation of the binding of thyroid stimulating hormone to receptor sites in normal and pathological human thyroid tissues. University of London, 1983. pp 368-96. Thesis for the degree of Doctor of Philosophy.
13. Kermode JC, Thompson BD. Iodination of thyroid stimulating hormone for receptor-binding studies with human thyroid membranes: effects of specific activity and method of iodination. *J Endocrinol* 1980; 84: 439-47.
14. Beall GN, Chopra IJ, Solomon DH, Kruger SR. Serum protein inhibition of thyrotrophin binding to human thyroid tissue. *J Clin Endocrinol Metab* 1978; 47: 967-73.
15. Rees Smith B, Hall R. Measurement of thyrotrophin receptor antibodies. *Methods Enzymol* 1981; 74: 405-20.
16. de Bruin TW, Van der Heide D. Anti-thyrotrophin receptor antibodies in Graves' disease as demonstrated directly by immunoprecipitation assay. *Acta Endocrinol (Copenh)* 1983; 102: 49-56.
17. Pekonen F, Makinen T. The interaction of thyrotrophin binding inhibiting immunoglobulins with high and low affinity TSH receptors. *Acta Endocrinol (Copenh)* 1983; 251 (Suppl): 33-7.