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A Sensitive Human Thyroglobulin RIA to Define Clearly the Presence or Absence of Functioning Thyroid Tissue

Irene Lemish, Francis Bennett, Clayton Martin, Anthony Warwick, and Michael Quinlan

Sir Charles Gairdner Hospital, Queen Elizabeth II Medical Centre, Nedlands, Western Australia, 6009

This paper describes a practical procedure to establish a sensitive radioimmunoassay for the measurement of human thyroglobulin (HTg) in serum. The assay enables a clear distinction to be made between levels of HTg indicating the presence or absence of functioning thyroid tissue. It compares the use of HTg serum levels with the conventional I-131 scintigram as a marker for recurrent thyroid cancer. In addition, a simple and sensitive radioimmunologic assay is described for the determination of serum antithyroglobulin autoantibodies, the presence of which may give falsely elevated HTg levels.

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Human thyroglobulin (HTg) is a 19S iodoglycoprotein of high molecular weight (660,000), found in measurable levels in the peripheral blood of euthyroid subjects. Since functioning thyroid tissue is the only source of HTg in peripheral blood, thyroid-cancer patients who have had all normal and malignant thyroid tissue removed should have no circulating HTg. Thus, the presence of HTg is a simple and specific marker of recurrent thyroid malignancy, and now provides a well-established method for the management of patients with thyroid cancer. Comparisons have been made between HTg levels and the I-131 image in patients who have undergone thyroid ablation for differentiated thyroid cancer (*1*,*2*). This study includes a further comparison of data on 30 patients over a period of 2 or more years.

MATERIALS AND METHODS

Patients. All patients had undergone treatment for differentiated thyroid carcinoma and had been receiving thyroid hormone replacement. This was discontinued and replaced with tri-iodothyronine for 3 wk, followed by a further 2 wk during which no thyroid hormone was given. Blood was then taken from each patient to estimate HTg and antithyroglobulin autoantibody levels, and concurrently an I-131 whole-body image was performed.

Purification of HTg. Extraction, isolation, and purification of HTg was based on a modified method of Van Herle (*3*). About 20 g of postmortem human thyroid tissue was mixed in a blender with two volumes of PBS (0.007*M* phosphate, 0.15*M* sodium chloride, pH 7.0) and the mixture stirred for 18 hr at 4°C. Tissue debris was removed by gauze filtration, followed by centrifugation of the filtrate at 12000*g* for 15 min. Ammonium sulfate (3.5*M*) was added slowly to the stirred supernatent up to a final concentration of 1.5*M*. The solution was centrifuged at 12000*g* for 15 min, and the supernatent treated with ammonium sulfate to a final concentration of 1.8*M*. All procedures were carried out at 4°C. After standing for 3 hr, the preparation was centrifuged and the precipitate redissolved in PBS before being concentrated in ultracentrifugation cones. The concentrate (3 ml) was applied to a Sephadex G200 column 3.5 × 35 cm) and eluted with PBS. Fractions (3.5 ml) immediately following the void volume (Fig. 1A) were pooled and concentrated using Amicon cones. The product was chromatographed on a Concanavalin A Sepharose 4B column by the method of Ochi et al. (*4*). Concanavalin A Sepharose (10ml) was equilibrated with

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For reprints contact: Irene Lemish, Sir Charles Gairdner Hospital, Queen Elizabeth II Medical Centre, Nedlands, West Australia 6009.

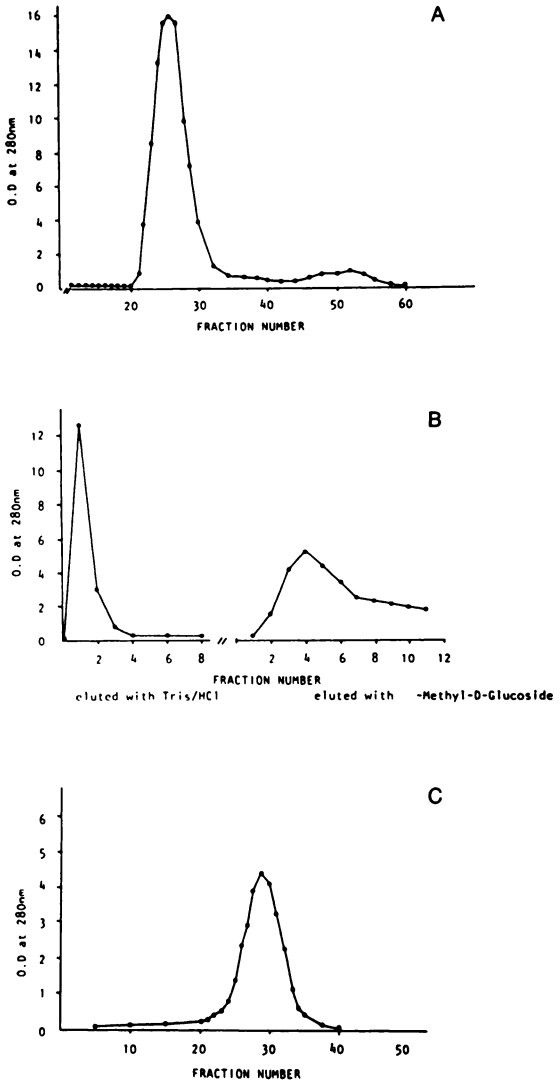

FIG. 1. (A) Separation of crude protein (3 ml) on Sephadex G 200 column; (B) Purification of HTg on ConA-Sepharose column; (C) Final purification of HTg on Sepharose 4B column.

Tris/HCl buffer (0.1*M* Tris, 0.1*M* sodium chloride, $10^{-3}M$ magnesium chloride, pH 7.6) and this buffer was passed through the column at a flow rate of 40 ml per hour to elute the nonglycoprotein material. Early fractions contained a white protein (Fig. 1B). Glycoproteins were eluted with 10% methyl-D-glucoside in Tris/HCl buffer (12 × 3.5 ml fractions). Fractions were pooled and concentrated using Amicon cones. When this concentrate was applied to a Sepharose 4B column (40 × 2.1 cm) and eluted in 0.2*M* phosphate buffer, pH 7.0, a single protein peak was observed (Fig. 1C). Peak fractions were pooled, concentrated, and aliquots (of known protein concentration) were lyophilized in glass ampoules, sealed, and stored.

Protein in the column fractions was measured by UV absorption at 280 nm; protein concentration in the final product was measured by the method of Lowry, as

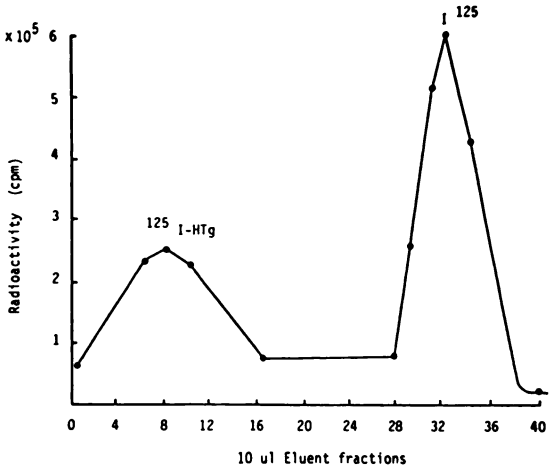

FIG. 2. Elution profile of I-125 HTg on Sepharose 6B.

modified by Hartree (*5*). The Lowry method measurements correspond well with dry-weight analysis, although the results are significantly higher than protein measured by the Ponceau-S method.

The purity of the HTg was assessed by:

1. The formation of a single band between the albumin and alpha region, when the protein was electrophoresed on cellulose acetate and stained with Ponceau-S dye.

2. The formation of a single precipitation line when the protein was immunoelectrophoresed against a serum containing antithyroglobulin. When the protein was immunoelectrophoresed against antibody to whole human serum, no precipitin line was observed, indicating that the preparation contained no serum proteins.

3. The formation of a single protein band in the region of pH 4.5 when the protein was electrophoresed in a pH gradient (isoelectrofocusing technique). HTg has a known isoelectric point of pH 4.5.

Preparation of I-125 labeled HTg. Five micrograms of purified HTg were labeled with I-125 (~37 MBq) by the lactoperoxidose technique (*6*). Free iodine was removed on a Sepharose 6B column (30 × 1 cm) eluted with 2% bovine serum albumin in 0.05*M* phosphate-buffered saline (PBS) at pH 7.4 (Fig. 2). A specific activity of about 0.1 mCi/μg was obtained in the ½-ml fraction containing the highest I-125 incorporation. This fraction (diluted 1:40 with PBS containing 0.001*M* EDTA Na) was used in all experiments. A 4-day incubation of freshly prepared I-125 HTg with excess antiserum caused the second antibody to precipitate 90% of the total radioactivity. Subsequently there was a loss of I-125 HTg immunoreactivity with time. Accordingly I-125 HTg was prepared fresh for every assay, since if this was not done elevated levels of HTg were obtained for patients' sera.

Preparation of antiserum. Antisera to HTg were raised in rabbits by multiple-site intradermal injections of a mixture of purified HTg (5 mg in 0.2 ml saline), com-

plete Freund's adjuvant (0.7 ml), and killed bacilli (0.1 ml) (*7*).

The rabbits received booster injections after 12 wk, and serum containing antithyroglobulin antibody was harvested after a further 2–4 wk. The antiserum was used in a final assay dilution of $1:10^6$, which bound 60–70% I-125 HTg.

Measurement of HTg.

1. The following reagents were added to conical plastic centrifuge tubes: (a) 200 μl I-125 HTg in PBS with 0.001*M* EDTA Na, giving a total count in each tube of ~20,000 cpm; (b) 200 μl unlabeled HTg standards ranging from 0–500 ng/ml in fetal calf serum (in triplicate), or 200 μl test sera (in duplicate); (c) 100 μl of a 1:200,000 dilution of antiserum in PBS with 0.001*M* EDTA Na.

2. The contents of the tubes were vortex-mixed and incubated at 4°C for 4 days.

3. The HTg-antibody complex was precipitated by the addition of (a) 100 μl carrier rabbit serum diluted 1:100 in PBS; (b) 100 μl donkey anti-rabbit gamma globulin* diluted 1:12 in PBS; and (c) 100 μl of 16% polyethylene glycol in PBS, with immediate mixing.

4. The tubes were allowed to stand for a minimum of 15 min before centrifuging at 3,000 rpm for 20 min. The precipitate was washed in 0.5 ml of PBS and counted in a gamma spectrometer. Results were calculated on a computer programmed after Burger et al. (*8*).

Determination of antithyroglobulin autoantibodies. All sera were screened for antithyroglobulin autoantibodies by a modifed method of Peake et al. (*9*).

A 2-μl aliquot of serum was pipetted into conical tubes using an automatic micropipettor with an accuracy of 2 μl ± 0.12 (s.d.).

This was incubated for 4 days with 200 μl of I-125 HTg PBS giving ~120,000 cpm per tube.

The complex of antibody and I-125 HTg was then precipitated with 15 μl of 12.5% *Staphylococcus aureus* protein A in PBS. The tubes were centrifuged at 3000 rpm for 20 min, and the pellet was washed with 1 ml PBS and counted in a gamma spectrometer. Nonspecific binding was determined by the addition of 2 μl fetal calf serum in place of the test serum. All counts not specifically bound were subtracted from all test-serum counts before calculating the percentage of immunoprecipitable I-125 HTg bound.

This method of measuring antithyroglobulin autoantibodies was compared with the tanned red-cell hemogglutination test†.

RESULTS

Standard curve. A typical standard curve for HTg in fetal calf serum is shown in Fig. 3. A similar curve was obtained when standards were prepared in 7% bovine serum albumin in PBS.

Theoretical sensitivity. The least detectable concentration was determined, using the calculated error at zero concentration. Two standard deviations were subtracted from mean counts at zero point, and the corresponding HTg concentration read off the standard curve. In ten assays it ranged between 0.5 and 0.7 ng/ml.

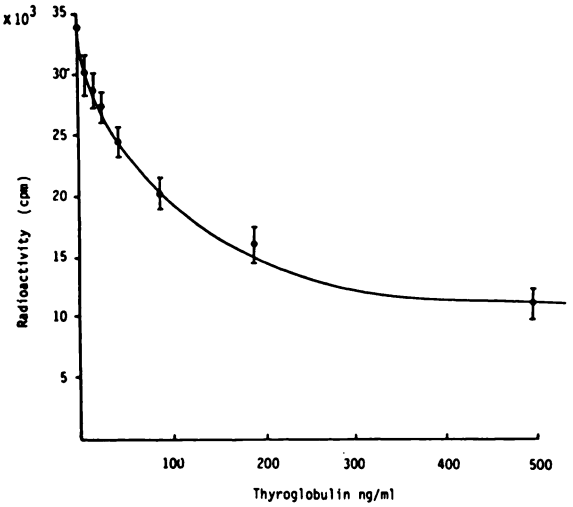

FIG. 3. Standard curve of thyroglobulin RIA.

Precision and reproducibility. The precision of the assay was determined by calculating the coefficient of variation (CV). The intraassay CV was calculated on 30 samples ranging from 1.4–246 ng/ml, and 30 samples ranging from 0.5–4 ng/ml, giving coefficients of variation of 8.1% and 2%, respectively.

The interassay CV of 10 samples (range 1–151 ng/ml), measured in three separate assays, was 9.5%.

Recovery experiments. Thyroglobulin concentrations of 50 and 100 ng/ml, respectively, were added to serum samples containing 1.0 ng/ml, giving theoretical concentrations of 51 ng/ml and 101 ng/ml. Using ten determinations in each, mean HTg concentrations of 54 ng/ml and 110 ng/ml were obtained, corresponding to mean recoveries of 103% and 109%.

Specificity. There was no displacement of I-125 HTg from antibody by T_4 and T_3 (10 μg/tube) under normal assay conditions.

Quality control. A human serum known to be free of HTg was set up with each assay as control. This serum exhibited a range of 0.5–2 ng/ml and the assay results were not accepted if this control serum showed greater than 2 ng/ml.

Normal reference ranges. A normal reference range of 4–30 ng/ml was established from 68 female and 49 male subjects having no antithyroglobulin autoantibodies, in the age range 20–45 yr.

No significant difference ($0.1 < p < 0.2$) was found between male and female in our study. Other workers have reported no significant difference between male and female (*4*), and that an increase in HTg levels is seen with respect to age. This increase is significant only in females of > 40 yr of age (*10*).

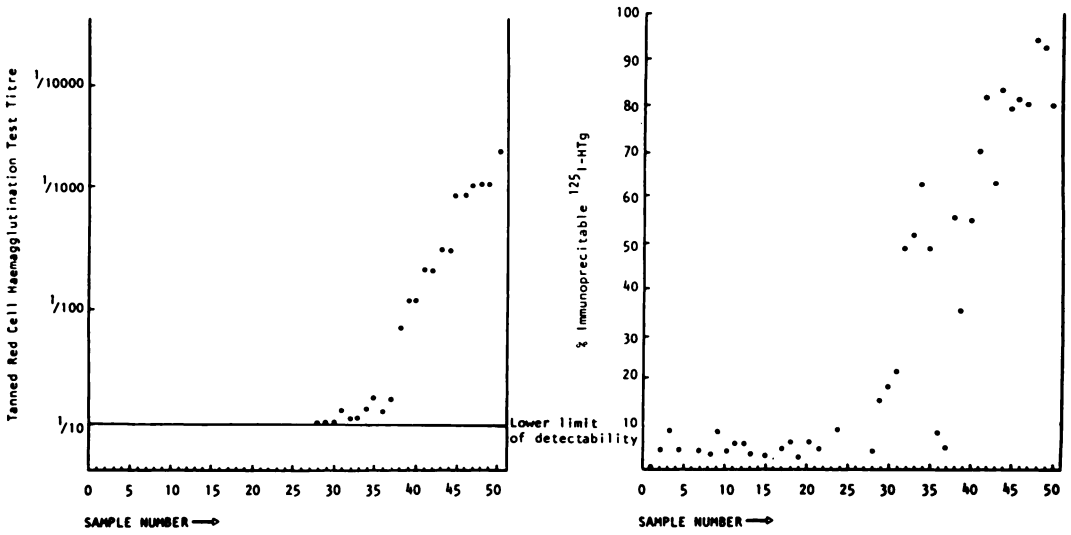

FIG. 4. Comparison of Tanned Red-Cell Hemagglutination Test and Radioimmunologic Assay.

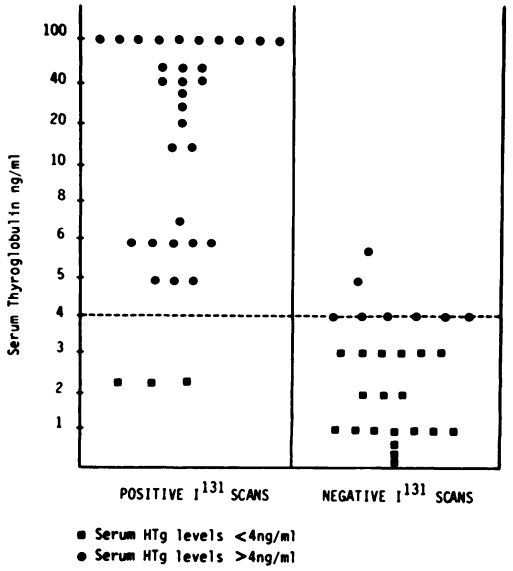

FIG. 5. Comparison of serum thyroglobulin levels with I-131 images.

In our study it was observed, but not statistically validated, that both male and female patients in the 40–45 yr range had HTg levels comparable with those of patients in the 20- to 45-yr range. (There were too few samples in the >40-yr group for statistical validation.)

Athyroid patients. An athyroid range was established on an initial study of 23 males and 31 females (having no antithyroglobulin autoantibodies) who had undergone total thyroidectomy. These patients showed no evidence of residual functioning thyroid tissue both clinically and upon I-131 imaging, and yielded serum thyroglobulin levels ranging from 0.5–2.5 ng/ml.

This nonspecific binding presented a clear distinction with respect to the lower end of the normal reference range.

Stability of RIA materials: All freeze-dried HTg stocks were in use for more than 4 yr and found to be stable. Frozen stocks of HTg antiserum were stable for several months. I-125 HTg was prepared fresh for each assay and used on the same day. The sodium iodide (I-125) used for iodinations was used within 4 wk of the reference date.

Antithyroglobulin auto-antibodies. A total of 146 sera from patients undergoing thyroid studies were imaged for autoantibodies by both the radioimmunologic (RI)

TABLE 1. HTg LEVELS (ng/ml). COMPARISON OF RESULTS

Age of 125-I-HTg days	Sample number → 1	2	3	4	5	6	7	8	9
0	1.1 ± 0.01	13 ± 0.2	2 ± 0.01	5 ± 0.06	42 ± 0.1	300 ± 0.07	0.9 ± 0.02	5.0 ± 0.02	0.5 ± 0.06
3	15 ± 0.02	24 ± 0.1	19.5 ± 0.02	29 ± 0.1	150 ± 0.1	7500 ± 0.06	11.5 ± 0.05	19.5 ± 0.03	10.5 ± 0.1
5	15 ± 0.1	25 ± 0.2	22 ± 0.01	37 ± 0.1	160 ± 0.2	7500 ± 1	9 ± 0.06	18.5 ± 0.02	19.5 ± 0.1
7	15.7 ± 0.05	25 ± 0.1	28 ± 0.1	38.5 ± 0.06	150 ± 0.1	7500 ± 1	11 ± 0.05	19.5 ± 0.2	13.2 ± 0.1
10	17.3 ± 0.1	23 ± 0.2	25 ± 0.3	40 ± 0.3	190 ± 0.2	7500 ± 6	13.5 ± 0.1	15.8 ± 0.1	17 ± 0.1
Inter-assay C.V. between Day 3 and Day 10.	5%	3%	13%	13%	9%	8%	18%	2%	30%

and tanned red-cell hemagglutination (TRC) technique. There was a significant correlation r = 0.58 (p = 0.001) between the two techniques (Fig. 4). The 44 sera with a positive titre by the T.R.C. method were positive by the RI method (10–90% I-125 HTg bound). There were 30 sera negative by the TRC and positive by the RI method. This is a reflection of the increased sensitivity of the RI method. The remaining 72 sera were negative for antibodies by both methods.

Euthyroid range. The radioimmunologic antithyroglobulin autoantibody assay in euthyroid subjects gave a reference range of 0–6%. Values over 6% were considered to indicate the presence of HTg autoantibodies.

Autoantibody levels of titer 1:80 by means of the TRC method are considered clinically significant. This corresponds to 40% antibody bound in our radioimmunologic method.

Comparison of HTg levels and I-131 whole-body images. After establishing the athyroid range, a comparison between serum HTg levels and I-131 whole-body images was made on an additional group of 30 patients who had undergone thyroidectomy and radioactive ablation for thyroid cancer.

The types of thyroid cancer exhibited were well-differentiated: follicular (11 patients), papillary (13), mixed follicular/papillary (6).

This study involved a total of 60 I-131 images and 60 serum HTg measurements, conducted over a 2-yr period after thyroidectomy (Fig. 5). All patients exhibited TSH levels (20–133 μU/l) well above the normal reference range (2–6 μU/l) before imaging and serum assaying.

Although HTg levels between 2.5 ng/ml and 4 ng/ml indicate the presence of thyroglobulin, they were not scored as thyroglobulin-positive, since these levels are not considered clinically significant in patient management. For the purpose of this comparison study levels of $\geq$4 ng/ml were scored as thyroglobulin-positive.

Thirty-five sera were positive for HTg, and 28 of them had concurrent positive I-131 images. Nineteen showed no evidence of recurrence either by image or serum HTg. However, six studies showing negative images were found to have HTg levels of 4 ng/ml. On further follow-up, five of these patients showed decreased HTg serum levels and negative I-131 images. The remaining patient showed a marked increase in HTg levels along with positive I-131 images. These results give our assay a high degree of specificity (0.9) in the diagnosis of recurrent thyroid cancer, and compare favorably with current published data (*1*).

HTg autoantibodies measured in serum samples of these patients ranged from 0–14% by the RI method.

Discussion. The RIA described for measuring HTg was based on that of Van Herle et al. (*3*) with the following modifications: Polyethylene glycol (final concentration 2%) was introduced to bring the double-antibody precipitation reaction to completion within 15 min. The I-125 iodinated HTg was prepared by the milder lactoperoxidase method. It was found necessary to use freshly prepared I-125 HTg in this assay.

Increased values of serum HTg were obtained when 20 samples ranging from 0.2–300 ng/ml were reassayed under constant conditions using I-125 HTg ranging from 1 to 10 days old (Table 1).

The amount of I-125 HTg bound by antithyroglobulin antisera was seen to decrease when old I-125 HTg was used. Since the concentration of I-125 HTg remained unaltered, the decrease in the amount of I-125 HTg available for binding was attributed to loss of immunoreactivity. This decreased affinity between antigen (I-125 HTg) and antisera resulted in the reaction's mimicking the reduction of bound counts seen when increased amounts of unlabeled HTg are present in the serum, hence producing falsely elevated levels.

This decrease in immunoreactivity with time was quantitated by calculating the antigenic decay of the I-125 HTg, this being the difference between the theoretical decrease in counts with time due to physical decay of I-125 and the observed decrease in counts.

If the I-125 HTg is stable, the slope of the plot of the observed counts will match the slope of the I-125 decay

OBTAINED USING FRESH AND AGED I-125 HTg

10	11	12	13	14	15	16	17	18	19	20
2.2 ± 0.01	0.8 ± 0.04	3 ± 0.01	6 ± 0.07	4.9 ± 0.02	6 ± 0.1	0.2 ± 0.02	0.4 ± 0.05	0.8 ± 0.1	2 ± 0.1	2 ± 0.1
7.5 ± 0.01	9 ± 0.6	7.5 ± 0.01	7.6 ± 0.05	17.5 ± 0.06	20 ± 0.1	10.5 ± 0.1	8 ± 0.1	7.3 ± 0.1	10.5 ± 0.1	7.3 ± 0.1
6.2 ± 0.06	10.7 ± 0.1	12.5 ± 0.02	10.2 ± 0.03	18 ± 0.1	27.5 ± 0.1	18 ± 0.1	7.5 ± 0.1	9 ± 0.02	11.5 ± 0.1	7.5 ± 0.1
9.3 ± 0.05	12.5 ± 0.2	13 ± 0.1	12 ± 0.1	17.5 ± 0.1	23.1 ± 0.2	16.5 ± 0.2	6 ± 0.1	5.3 ± 0.07	6 ± 0.1	6.1 ± 0.1
14.2 ± 0.1	11 ± 0.2	15.5 ± 0.5	18.5 ± 0.1	22 ± 0.2	40 ± 0.4	21 ± 0.1	15 ± 0.2	12 ± 0.03	19 ± 0.5	18 ± 0.1
46%	14%	38%	51%	89%	35%	35%	4%	45%	76%	69%

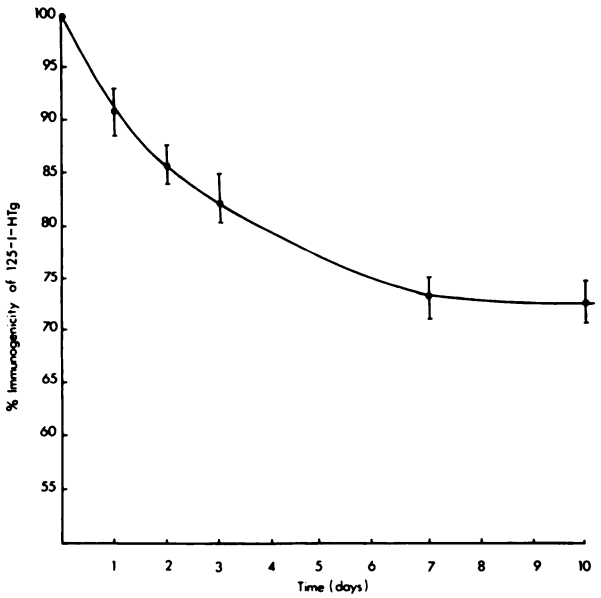

FIG. 6. Antigenic decay curve.

curve. Figure 4 represents a composite of 25 samples that were assayed in triplicate over a 10-day period.

The antigenic decay is expressed as a percentage of the theoretical maximum binding obtainable with a stable antigen for 0–10 days after iodination.

The loss of immunogenicity appears to be exponential. A rapid loss is seen between Day 0 and Day 3. This is confirmed by the increase in serum HTg levels seen in Table 1. The mechanism of this antigenic decay is unknown.

Whether the loss of antigenicity in this study was due to radiolytic decay or to fragmentation of the I-125 HTg is under investigation.

The observation of increased HTg values with time may explain why other workers (*4,11,12*) obtained higher normal ranges, and their inability to distinguish between euthyroid and athyroid levels.

Their apparent lack of sensitivity (Table 2) may be attributed to the loss of antigen (I-125 HTg) specificity with time, as the sensitivity of the assay is based on the high degree of specificity of the antigen for the antibody.

It has been well established that antithyroglobulin autoantibodies will interfere with the determination of HTg levels in the double-antibody precipitation RIA (*3,13*). Excluding samples on the basis of a positive TRC result has not proved adequate, since low titers of HTg antibodies—even below the limit of detection by the T.R.C. method—can affect the HTg RIA (*13*). The concurrent determination of HTg antibodies using freshly iodinated HTg and protein A as an immunoprecipitant provides a simple and sensitive radioimmunologic assay for screening serum samples being used for HTg determination.

Positive levels of autoantibodies (6–14%) were found in the ten serum samples that were used in the comparison study of HTg levels and I-131 whole-body images. The presence of these antibodies at these low levels did not appear to alter HTg levels significantly from a clinical point of view or spoil the good correlation between the two methods (specificity 0.9).

The results of the comparison of HTg levels with the I-131 image indicate the need for continual monitoring of HTg levels to ensure an accurate clinical picture, rather than relying on an isolated or random HTg level. This is evidenced by the follow-up change seen in the six patients showing initially negative I-131 images and positive HTg levels.

The results of this study clearly demonstrate the ability of this assay to distinguish HTg levels in subjects with normally functioning thyroid tissue from those in patients who are clinically athyroid. It demonstrates its value in the detection of residual or neoplastic thyroid tissue following thyroid ablation for the treatment of carcinoma.

TABLE 2. COMPARISON OF RESULTS OBTAINED BY VARIOUS AUTHORS FOR HTg RADIOIMMUNOASSAY

Author (Ref.)	% Normal Subjects with unmeasurable HTg	Assay sensitivity (ng/ml)	Normal range (ng/ml)	Mean (ng/ml)
Torrigani (*11*)	39	10	10–150	N.A.*
Van Herle (*3*)	26	0.8	1.6–20.7	5.1
Ochi (*4*)	56	10	10–180	N.A.
Lo Gerfo (*12*)	48	10	10–60	N.A.
Schneider (*14*)	N.A.	5*	5–40	15
Galligan (*1*)	1.9	1.25	1.25–38	11
This study	0	0.7	4–30	13

* N.A. = Data not available.

FOOTNOTES

* Wellcome.
† Thymune T test kit, Wellcome.

ACKNOWLEDGMENTS

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