Nonisotopic "Sandwich" Immunoassay of Thyroglobulin in Serum by the Biotin–Streptavidin Technique: Evaluation and Comparison with an Immunoradiometric Assay

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In this sequential assay, the thyroglobulin in serum binds to polystyrene beads coated with two mouse monoclonal antibodies. These beads then react with a rabbit polyclonal antibody, biotinylated sheep anti-rabbit IgG, streptavidin–horseradish peroxidase, and a peroxidase substrate to yield a colored product that is measured spectrophotometrically at 492 nm. The range of the standard curve is 1.6 to 100 μg/L. The detection limit of the assay is 1.2 μg/L. The interassay coefficient of variation is 14.0% at 6.2 μg/L, 5.7% at 32.7 μg/L; the intra-assay CVs range from 7.8% to 14.8%. The reference intervals are 2.7 to 42.1 μg/L for euthyroid persons and ≤5 μg/L for athyreotic patients not on thyroxin replacement therapy. Some autoantibodies to thyroglobulin cause thyroglobulin values to be falsely low. The concentration of autoantibodies is not correlated with the analytical recovery of human thyroglobulin. The coefficient of correlation between the measurement of thyroglobulin by this assay and by an immunoradiometric assay was 0.969 for 186 autoantibody-negative samples, 0.963 for 37 autoantibody-positive samples.

Human serum thyroglobulin has been measured by double-antibody radioimmunoassay (1–4) or solid-phase immunoradiometric assay (IRMA) (5–7); a few reports of enzyme immunoassays are now appearing in the literature (8, 9). The presence of autoantibodies to thyroglobulin has been a continuing problem in these assays. The IRMA procedures were developed because autoantibodies to thyroglobulin produced either falsely low or falsely high thyroglobulin values in the radioimmunoassays. Use of IRMA has not eliminated the autoantibody problem; however, it has succeeded in limiting it to only underestimation of the true thyroglobulin concentration (5, 6).

We have developed a solid-phase, "sandwich"-type, immunoassay for measuring thyroglobulin in which the biotin-streptavidin technique (10) is used and we have adapted a binding assay for detecting autoantibodies to thyroglobulin (11). We examined the effects of anti-thyroglobulin autoantibodies in our assay and compared our thyroglobulin results with those obtained by using a commercially available IRMA. Reference intervals were established for both euthyroid and athyreotic persons. We also measured thyroglobulin in a series of patients with various thyroid disorders.

Materials and Methods

Materials

Polyoxyethylene (20) sorbitan monolaurate (Tween 20), bovine serum albumin (cat. no. A-7030), sodium metabisulfite, hydrogen peroxide (300 g/L), the biotin-conjugated Fab'2 fragment of sheep anti-rabbit IgG, and human IgG covalently bound to agarose were obtained from Sigma Chemical Co., St. Louis, MO 63178. Bovine calf serum was from Pel-Freez Biologicals, Rogers, AR 72756. The horseradish peroxidase (EC 1.11.1.7)–streptavidin solution and 125I were from Amersham Corp., Arlington Heights, IL 60005. O-Phenylenediamine tablets were from DAKO Corp., Santa Barbara, CA 93103. Chloramine T was from Eastman Kodak Co., Rochester, NY 14650.

Ultrogel AcA 34 was from LKB Instruments, Inc., Gaithersburg, MD 20877. Bio-Gel P-60 was from Bio-Rad Laboratories, Richmond, CA 94803; Bio-Gel A-5 and Protein A–Sepharose CL-4B were from Pharmacia Inc., Piscataway, NJ 08854.

Phosphate-buffered isotonic saline contained phosphate at 10 mmol/L (pH 7.4) and NaCl at 150 mmol/L. Wash buffer contained 1 g of Tween 20 in 1 L of the phosphate-buffered saline. Working buffer contained 1 g of Tween 20 and 1 g of bovine serum albumin in 1 L of phosphate-buffered saline.

Subjects

Euthyroid. We used serum samples from 42 healthy volunteers (Mayo Clinic employees) to establish the normal reference interval: 19 men, ages 27 to 69 y (median, 39 y) and 23 women, ages 26 to 64 y (median, 37 y).

Athyreotic. We studied 59 serum samples from 56 patients with differentiated thyroid cancer that had been surgically resected and radio-ablated. These patients included 15 boys and men, ages 15 to 65 y (median, 54 y) and 41 women, ages 20 to 70 y (median, 36 y). These samples were obtained while the patients were not being treated with thyroid hormone replacement therapy, in the period before 131I whole-body scanning. There was no radiological or clinical evidence of recurrence of tumor at the time the serum was collected. The samples were stored at −20 °C until assayed.

Preoperative. Serum samples were collected from 102 patients prior to thyroid surgery for malignant (n = 38) or benign (n = 64) nodules or for autoimmune thyroid disease (n = 3).

Methods

Purification of thyroglobulin. Human thyroglobulin was isolated as described (12). Normal human thyroid glands, obtained at operation, were minced and incubated for 12 to 16 h at 4 °C prior to centrifugation. The supernatant fluid was dialyzed against KCl–phosphate buffer and purified on a column of Bio-Gel A-5. The thyroglobulin-containing fractions were pooled, dialyzed against phosphate-buffered saline, and stored at −70 °C in separate small portions.

Standards of thyroglobulin with concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 μg per liter were prepared by diluting a 1.8 g/L stock solution in bovine calf serum. The standards were stored frozen at −20 °C.

Preparation of antibodies. Monoclonal antibody 30a was prepared in B10.S mice immunized by injection of human
thyroglobulin (20 μg in complete Freund's adjuvant) into the hind footpads. Three days after an intravenous booster injection of 10 μg of antigen in phosphate-buffered saline, the spleens were removed and fused with F/O myeloma cells (13). To test the culture supernates for the presence of relevant antibody, we used a solid-phase immunoassay with sheep anti-mouse-β-galactosidase conjugate (Bethesda Research Laboratory, Gaithersburg, MD 20877) or a radioimmunoassay with 125I-labeled Protein A (New England Nuclear, Boston, MA 02118). Hybridomas of interest were cloned in limiting dilution cultures with BALB/c spleen cells as feeder layer cells. Ascitic fluid was produced in (BALB/c × B10.S)F1 mice and collected as described (14).

After overnight dialysis against phosphate-buffered saline the ascitic fluid was purified over a Protein A—Sepharose CL-4B column. The immunoglobulin fractions were pooled, concentrated in an ultrafiltration cell (Amicon Corp., Danvers, MA 01923), and dialyzed. Monoclonal antibody 30a was isotype to IgG1 with κ light chains by a solid-phase immunoassay by use of antisera from Southern Biotechnology Associates, Inc., Birmingham, AL 35226.

Monoclonal antibody 31.4 was produced in BALB/c mice given four intraperitoneal 50-μg injections of human thyroglobulin. After fusion with NS-1 cells and multiple screenings for suitable hybridomas by using 125I-labeled thyroglobulin, 10 clones were passed into mice. The resulting ascitic fluids were purified by precipitation with 50% saturated ammonium sulfate (final concentration) followed by chromatography on a 3 × 80 cm column of Ultrogel AcA 34 equilibrated with phosphate-buffered saline. The fractions that contained high immunoglobulin concentrations and little or no albumin (as determined by single immunoradiodiffusion) were pooled, and the protein content was determined, and the solution was stored at −70 °C in separate small portions. Monoclonal antibody 31.4 was isotype to be an IgG1 with κ light chains by using antisera from Litton Bionetics, Kensington, MD 20795.

A polyclonal antibody was produced in New Zealand White rabbits. Multiple subcutaneous injections of a human thyroglobulin preparation resulted in an antiserum with a sufficiently high titer on the third harvesting. Purification of the antibody was similar to that of 31.4 except that precipitation with 40% saturated (final concentration) ammonium sulfate was done before chromatography. In addition, antibodies to human immunoglobulins were removed by affinity chromatography on 500 μL of human IgG covalently bound to agarose for each 3 mL of purified rabbit antibody. The flow-through was collected and used in the assays.

Coating of solid phase. Polystyrene beads (6.4 mm, grade 2, frosted finish) purchased from Clifton Plastics, Inc., Clifton, Heights, PA 019018, were used as the solid phase. They were coated overnight at room temperature with a mixture of purified monoclonal antibodies 30a and 31.4 at a final concentration of 0.5 μg per bead in carbonate–bicarbonate buffer (pH 9.6). The remaining binding sites on the beads were blocked by incubation with bovine serum albumin solution (50 g/L in phosphate-buffered saline) for 2 h at 37 °C. The beads were stored at 4 °C for 6 to 8 weeks in working buffer without measurable loss of activity.

Thyroglobulin assay. Duplicate 200-μL portions of standards, controls, and patients' serum were pipetted into 12 × 75 mm polystyrene tubes. Then, an antibody-coated bead and 500 μL of working buffer (containing nonimmune mouse serum; 10 mL/L) were added. The tubes were incubated overnight at 4 °C, then washed with three 1-mL portions of wash buffer. Rabbit anti-thyroglobulin antibody (diluted in working buffer to 1 μg/200 μL) was added to the tubes, which then were incubated at room temperature for 3 h on a horizontal rotator. The tubes were washed as above and incubated for 2 h at room temperature with 200 μL of a 1:2500 dilution of sheep anti-rabbit IgG–biotin conjugate. After another wash step, the tubes were incubated for 30 min at 37 °C with 200 μL of a 1000-fold dilution of horseradish peroxidase–streptavidin. The tubes were washed and 200 μL of fresh o-phenylenediamine substrate was added. The reaction was stopped at 10 min by addition of 1 mL of HCl solution (1 mol/L), and the absorbance at 492 nm was measured. Any sample found to contain more thyroglobulin than 100 μg/L was diluted 10-fold (20 μL of sample and 180 μL of bovine calf serum) and assayed again.

Controls. To samples of blood-bank serum (converted from plasma), we added a thyroglobulin standard preparation obtained from Accurate Chemical and Scientific Corp., Westbury, NY 11590, to achieve concentrations of about 5, 15 to 20, and 35 to 40 μg/L.

Determination of autoantibodies to thyroglobulin. All patients' samples were screened for the presence of anti-thyroglobulin autoantibodies by using a modification of the double-antibody radioimmunoassay developed by Bolland et al. (11). In brief, 200 μL of 125I-labeled thyroglobulin (15 000 to 20 000 cpm) was incubated for 30 min at 37 °C with 10 μL of serum. We then added 200 μL of goat anti-human serum and 100 μL of polyethylene glycol solution (90 g/L), and continued the incubation overnight at 4 °C. Two milliliters of phosphate-buffered saline was added and the mixture was centrifuged (500 × g, 15 min, 4 °C) to collect the immunoprecipitates. To calculate percentage binding, the number of counts bound was divided by total counts and this quotient was multiplied by 100. Three control pools (n = 25) with medium binding values of 4%, 20%, and 60% were included in each assay.

Binding of 7% or more of the radiolabeled thyroglobulin was considered to be positive for the presence of autoantibodies. Analytical recovery of added thyroglobulin (20 μg/L) was measured on all samples positive for autoantibodies; recovery of 70% or more was regarded as adequate support for reporting the result. Bovine calf serum with thyroglobulin added to the same concentration was included as a control in the recovery determinations.

IODINATION OF THYROGLOBULIN (FOR AUTOANTIBODY SCREEN). Thyroglobulin was iodinated by a modification of the procedure of Hunter and Greenwood (15). One milliliter of 125I and 10 μL of a 0.5 g/L Chloramine T solution were added to 22 μg of thyroglobulin in 50 μL of phosphate buffer (100 mmol/L, pH 7.5). Contents of the vial were vortex-mixed for 30 s and then 25 μL of a 1 g/L sodium metabisulfite solution was added. The reaction mixture was transferred to a Bio-Gel P-60 column. We eluted with a phosphate buffer containing bovine serum albumin, 10 g/L, and collected 5-mL fractions. The radiiodinated peak fraction(s) were diluted to 20 mL in this phosphate–bovine serum albumin buffer to obtain a specific activity of 15 to 20 μCi/μg. The labeled thyroglobulin was stored at 4 °C for up to eight weeks.

Results

Standard Curve

Figure 1 depicts a composite of six standard curves for thyroglobulin. The seven concentrations used in the assay
represented doubling dilutions from 100 to 1.6 µg/L. For data-reduction purposes, a best-fit linear curve was used from 1.6 to 50 µg/L, and point-to-point interpolation was used for values between 50 and 100 µg/L.

The detection limit calculated from 16 consecutive assays was 1.2 µg/L (2 SD from the zero standard). No apparent "hook" effect due to antigen excess was noted when the thyroglobulin standard was tested at concentrations from 10 µg/L to 1 g/L.

The results of linearity studies, performed by serially diluting three autoantibody-negative patients' samples with bovine calf serum (Table 1), were acceptable, with the ratio of observed to expected thyroglobulin values ranging from 0.86 to 1.25 (n = 14; mean ± SD, 1.06 ± 0.12).

Reference Intervals

The 95% normal reference interval estimated from 42 euthyroid volunteers was 2.7 to 42.1 µg/L (mean ± SD, 18.2 ± 9.9 µg/L). This agrees well with previously reported values (3, 5, 16). There was no significant sex-related difference: mean 18.5 (SD 11.6) µg/L, for females 17.8 (SD 8.5) µg/L. Two (4.8%) of these 42 serum samples contained very low ratios (8% and 7% binding) of autoantibodies to thyroglobulin; these samples had thyroglobulin values of 3.1 and 21.9 µg/L with recoveries of 80% and 82%, respectively. Because the thyroglobulin concentrations in these two samples were similar to the other values and the recoveries of added thyroglobulin were adequate, these results were included in the study.

In 32 of the 59 serum samples collected from 56 athyreotic patients, thyroglobulin was undetectable (<1.6 µg/L). However, 16 of these 32 samples contained autoantibodies to thyroglobulin, with bindings of 7% to 53% (mean ± SD, 24.2% ± 16.5%); 12 of the 16 showed recoveries of 70% or greater and the remaining four had recoveries of 12%, 31%, 51%, and 55%.

Of the 27 samples from athyreotic patients that did have measurable thyroglobulin values (≥1.6 µg/L), eight were below the cutoff level of 5 µg/L. In the remaining 19 samples the thyroglobulin concentration was 5 to 193 µg/L by solid-phase immunoassay (mean, 24.4 µg/L; median, 9.8 µg/L). Nine of these 19 samples also were assayed by IRMA; seven contained thyroglobulin ≥5 µg/L.

The medical histories of the 56 athyreotic patients revealed no differences, at the time of thyroid operation, with regard to type or size of tumor, presence of metastatic lymph nodes, or invasiveness of the tumor to explain the postoperative thyroglobulin results.

Preoperative Samples

We collected 102 serum samples from patients before operation for thyroid cancer (n = 38), nodules (n = 49), goiter (n = 12), or autoimmune disease (n = 3). The various histological types represented in the cancer group and the mean concentrations of serum thyroglobulin were as follows: four medullary, 18.5 µg/L; 24 papillary, 92.0 µg/L; two anaplastic, 233 µg/L; four recurrent or metastatic disease, 369 µg/L; and four follicular, 545 µg/L. Five of these 38 samples contained autoantibodies to thyroglobulin. Of the remaining 33, 17 had increased thyroglobulin concentrations ≥42.1 µg/L. The mean thyroglobulin concentration in the cancer group as a whole was 168 µg/L, similar to that in patients with nodules (192 µg/L) or goiter (210 µg/L).

Of the 47 autoantibody-negative samples from patients with nodules, 32 had thyroglobulin concentrations outside the normal reference interval. Only one of the 10 autoantibody-negative samples from goiter patients had a thyroglobulin value <42 µg/L preoperatively.

Precision

The coefficients of variation (Table 2) derived from three thyroglobulin control pools ranged from 7.8% to 14.8% within assay (n = 20) and from 5.7% to 14.0% between assays (n = 8).

| Table 1. Linearity Studies of Three Autoantibody-Negative Patients' Serum Samples |
|-----------------------------|-----------------------------|-----------------------------|
| Dilution | Expected | Observed | Observed/Expected, % |
| 0 | 23.7 | 23.7 | 100 |
| 1:2 | 11.9 | 13.2 | 111 |
| 1:4 | 5.9 | 6.1 | 103 |
| 1:8 | 0.4 | 2.7 | 90 |
| 0 | 90.1 | 90.1 | 100 |
| 1:2 | 45.1 | 48.9 | 108 |
| 1:4 | 22.5 | 27.4 | 122 |
| 1:8 | 11.3 | 13.3 | 118 |
| 1:16 | 5.6 | 5.4 | 96 |
| 0 | 81.7 | 81.7 | 100 |
| 1:2 | 40.9 | 43.4 | 106 |
| 1:4 | 20.4 | 23.7 | 116 |
| 1:8 | 10.2 | 12.7 | 125 |
| 1:16 | 5.1 | 4.4 | 86 |

*Overall mean ± SD: 105.8% ± 11.6%.

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<th>Table 2. Precision of the Assay</th>
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*Coefficient of variation (CV).
Comparison with IRMA

For comparison of the results obtained with our solid-phase immunoassay with those obtained with a commercial IRMA kit (Damon Biotech, Needham Heights, MA 02194), correlation coefficients were 0.969 with 186 autoantibody-negative serum samples and 0.963 with 37 autoantibody-positive serum samples (Figure 2).

Screening for Autoantibodies to Thyroglobulin: Radioimmunoassay and Hemagglutination

The quantitative hemagglutination test ("Sera-Tek"; Miles Laboratories, Inc., Elkhart, IN 46515) used in our institution for the routine testing of patients' serum samples for anti-thyroglobulin autoantibodies was compared with a sensitive radioimmunoassay procedure that we adapted for our laboratory. When 122 serum samples were tested by both methods, none was positive by hemagglutination and negative by radioimmunoassay but 22 (18.0%) were negative by hemagglutination and positive by radioimmunoassay. The percentage of immunoprecipitable 125I-labeled thyroglobulin in these samples ranged from 7% to 36% (mean, 15.1%; median, 12.5%). Of the remaining 100 samples tested, 79 were negative and 21 were positive by both methods.

Recovery of Thyroglobulin in Autoantibody-Negative and Autoantibody-Positive Serum Samples

The recoveries in the 26 autoantibody-negative serum samples ranged from 54% to 123% (median, 96%; mean, 91.4%) (Figure 3). The 66 autoantibody-positive samples, with binding of radiolabeled thyroglobulin from 7% to 84%, displayed recoveries of 3% to 125% (median, 70.5%; mean, 70.7%). There was no correlation between percentage of autoantibody binding and recovery of added thyroglobulin.

Analytical recovery of thyroglobulin added at two concentrations was determined in three serum samples from euthyroid patients. Thyroglobulin was added, in amounts representing 10 μg/L and 20 μg/L, to samples for which the endogenous thyroglobulin values were 15.6, 8.1, and 33.4 μg/L. The recoveries ranged from 97% to 112%, with no consistent differences in recovery between addition of 10 μg/L and 20 μg/L.

Discussion

This solid-phase immunoassay for thyroglobulin exploits the extremely high affinity between biotin and streptavidin (dissociation constant, 10^-15 mol/L) and the amplification effect of multiple-site binding to achieve a high sensitivity. In addition, we selected two monoclonal antibodies from 17 developed at our institution and combined them with a polyclonal antibody to produce an assay with the required sensitivity.

Although relatively straightforward and easy to perform technically, the traditional radioimmunoassay for thyroglobulin has been particularly susceptible to interference from autoantibodies in the sample. The IRMA was developed to eliminate this effect. However, the problem continued but was decreased to just underestimation of thyroglobulin values (6). The same is true for our solid-phase immunoassay.

Autoantibodies are usually measured by the tanned erythrocyte agglutination technique, and positive samples are excluded from the thyroglobulin assay. Schneider and Pervos (2) and Bodlaender et al. (11) have shown that autoantibodies present at concentrations not detectable by the agglutination technique still can interfere in the thyroglobulin assay. Therefore, it is necessary to use a sensitive method such as that developed by Bodlaender et al. (11) for screening patients' samples for autoantibodies.

We modified the Bodlaender et al. (11) screening procedure and compared it with the hemagglutination test routinely used in our institution. When 122 samples were assayed by both methods, 22 additional positives were detected by radioimmunoassay. This finding can be significant in the interpretation of thyroglobulin data because of the proportion of patients who have autoantibodies. We found the prevalence of autoantibodies measured by radioimmunoassay to be two (4.7%) of 42 euthyroid normals, 11 (10.8%) of 102 preoperative thyroidectomy patients, and 20...
(34.5%) of 58 athyreotic patients being monitored for recurrence of thyroid cancer.

In regard to the interference of autoantibodies in our solid-phase immunoassay, the recovery of added thyroglobulin was ≥74% in all but four of 26 autoantibody-negative samples; in these four samples, recoveries were <70% on both initial and repeat testing. (Similar recovery rates were found in additional serum samples from three of these four patients.)

The cutoff value of 5 μg/L that we established in patients with no residual thyroid tissue is similar to the value, 6.4 μg/L, reported by Falk et al. (4). The 18 patients (19 serum samples) whose serum contained thyroglobulin at ≥5 μg/L when the whole-body scans were negative have been followed for a mean of 18.9 months (range, 0 to 60 months). There are possible explanations for these increased thyroglobulin concentrations in some of the patients; in others the values are unaccounted for. Falk et al. (4) thought that such patients represent a high-risk group and should be monitored carefully for possible recurrence of their thyroid cancer.

As others have found, thyroglobulin concentration measured preoperatively cannot be used to distinguish thyroid cancer from benign nodules or goiter. The values within each of these three groups ranged from <1.6 to >1000 μg/L and the mean concentrations were similar—168, 192, and 210 μg/L.

The thyroglobulin results from our solid-phase immunoassay correlated well (r = 0.96) with those from a commercially available IRMA kit. Bayer and McDougall (16) also found a good correlation (r = 0.964) when they compared results with the kit with those by an IRMA developed at Stanford.

References