Novel Immunoradiometric Assay of Thyroglobulin in Serum with Use of Monoclonal Antibodies Selected for Lack of Cross-Reactivity with Autoantibodies

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A multisite immunoradiometric assay for measurement of serum thyroglobulin (Tg), designated Magnogel®-IRMA-Tg, has been developed, involving magnetic microbeads (Magnogel®). This assay is based on the use of five anti-Tg monoclonal antibodies (MAbs) directed against three antigenic regions on the Tg molecule that are not recognized by anti-Tg autoantibodies (aAbs). Four of these MAbs, directed against two antigenic domains, were coupled to the magnetic beads and were used to trap the serum antigen. Another MAb, directed against the third region, was iodinated and served as the labeled second antibody. The Magnogel-IRMA-Tg technique is reproducible, rapid, and sensitive (lower detection limit, 3 μg/L). The assay reliably measures serum Tg in the presence of anti-Tg aAbs.

The assay of thyroglobulin (Tg) in the serum is used in the follow-up of patients with thyroid diseases such as differentiated thyroid carcinoma and Graves' disease (1, 2). In the first case, the presence of Tg is indicative of metastasis after total ablation of the thyroid gland (1). In the second case, the evolution of the disease can be followed by monitoring the concentration of Tg in serum so as to decide whether or not to interrupt the treatment with synthetic anti-thyroid agents (2).

The numerous assays of Tg involving polyclonal antibodies (3–5) are limited by two major problems: considerable variability in the concentration of Tg determined (6) and, more importantly, interference from anti-Tg autoantibodies (aAbs) (7). Several investigators (8–10) have developed immunometric assays of Tg based on the use of monoclonal antibodies (MAbs). Kato et al. (11) developed an immunoenzymatic sandwich technique in which one MAAb was used as the trapping antibody and a second MAAb was coupled to peroxidase; these MAAbs showed little interference from aAbs.

Given that the Tg molecule displays several antigenic determinants, we quantified Tg in serum by a multi-site immunoradiometric assay (IRMA), using four anti-Tg monoclonal antibodies to trap the antigen and one iodinated anti-Tg MAAb as the second antibody. These five MAAbs recognize three different antigenic regions on the Tg molecule that are not recognized by anti-Tg aAbs (12).

Materials and Methods

Tg and sera. Pure Tg (13) was supplied by Dr. P. Carayon, School of Medicine, Marseilles, France. The serum samples were obtained from patients with thyroid disorders and from healthy subjects.

Anti-Tg monoclonal antibodies. We have produced in our laboratory 15 anti-Tg MAbs, which recognize six different antigenic regions on the human Tg molecule (14). Two of these regions are recognized by anti-Tg aAbs in the serum of patients with Hashimoto's thyroiditis, Graves' disease, and patients with thyroid carcinoma (12). For the present study, we used five of our anti-Tg MAbs directed against the three other antigenic domains on the Tg molecule not recognized by the aAbs in patients' sera.

Preparation of antibody-coated Magnogel. We mixed 1 mL of sediments commercial glutaraldehyde-activated Magnogel (Diagnostic Pasteur, Marne la Coquette, France) (15) with the antibody preparation: 0.5 mg each of MAbs 2, 5, and 13 and 1.5 mg of MAbs 4 in 1 mL of phosphate buffer (0.1 mol/L, pH 8.2). After incubating the antibody-coated beads for 16 h with gentle shaking, we allowed them to settle, removed the supernatant, and washed the beads with more phosphate buffer. Excess active groups were blocked by mixing the antibody-coated beads with an equal volume of glycine reagent, 0.4 mol/L in the phosphate buffer. After gently shaking the suspension for 3 h at room temperature, we removed any excess glycine and washed the beads with the phosphate buffer. The activated beads were stored as a 230 mL suspension in phosphate buffer containing 5 g of gelatin and 1 g of sodium azide per liter.

Preparation of anti-Tg MAbs labeled with 125I. Using the Chloramine T method, we iodinated 40 μg of purified MAAb (in 40 μL of 0.1 mol/L phosphate buffer, pH 7.5) with 1 mCi (37 MBq) of 125I. After adding 30 μL of Chloramine T reagent (0.33 g per liter of phosphate buffer), we shook the solution for 60 s, then stopped the reaction by adding 100 μL of Na2S2O5 solution (2.50 g per liter of phosphate buffer), followed by 100 μL of KI solution (10 g per liter of phosphate buffer). Unbound radioactivity was removed by passing the product through a PD-10 chromatographic column (Pharmacia, Uppsala, Sweden). We stored the iodinated MAAb in phosphate buffer (0.1 mol/L, pH 7.5) containing 1 g of bovine serum albumin per liter. The specific activity of the labeled MAAbs was 10 to 20 Ci per gram of antibody (dry weight).

Magnogel immunoradiometric assay for Tg. This assay technique makes use of the magnetic microbeads coated with trapping antibodies. In brief, Tg standard solution or an unknown serum sample is incubated with the antibody-coated Magnogel in tubes; unbound Tg is removed by turning the tubes upside down after having pulled the beads to the side, near the bottom of the tubes, with a magnetic bar. The tubes are then turned right side up again, iodinated second antibody is added, unbound 125I-labeled anti-Tg MAAb is removed magnetically, as before, and the radioactivity remaining bound to the beads in each tube is measured in a gamma counter.

The Tg assay procedure was as follows. Just before use, the antibody-coated Magnogel suspension was diluted 11.5-fold with phosphate-buffer (0.01 mol/L, pH 7.4) containing 0.15 mol of NaCl and 5 g of gelatin per liter. We mixed, by shaking for 3 h at room temperature, 300 μL of this suspension with 200 μL of Tg standard solutions (prepared

Received October 11, 1988; accepted December 22, 1988.
in phosphate-buffered isotonic saline containing 1 mL of Tween 20 per liter), 200 μL of serum sample, or, for the analytical-recovery experiments, 200 μL of a mixture of 100 μL of serum plus 100 μL of each Tg standard solution. Unbound Tg was removed magnetically, as described above, and the beads were washed in the phosphate-buffered saline/Tween 20 reagent. We then added to each tube 300 μL (200,000 counts/min) of 125I-labeled MAb 12 in phosphate-buffered saline containing 1 mL of Tween 20 and 5 mL of defatted milk per liter. After incubation for 2 h at room temperature with shaking, we washed the beads three times, then measured the radioactivity bound to the beads in each tube.

**Measurement of anti-Tg antibodies in patients' sera.** To detect aAbs, we used an anti-Tg IRMA kit (SB-AB-HTG-2, ORIS Industrie, Gif-sur-Yvette, France) and an indirect enzyme-linked immunosorbent assay described previously (12). In the latter, our criterion for the presence of the anti-Tg aAbs was an absorbance exceeding 1.0 at 405 nm for serum diluted 10-fold.

**Results**

Figure 1a illustrates a representative standard curve for the Magnogel-IRMA-Tg technique, covering a concentration range of 0 to 1000 μg/L. This curve is linear up to 100 μg/L (Figure 1b), and the lower limit of detection is 3 μg/L.

When serial twofold dilutions of sera with high Tg concentrations and free of anti-Tg autoantibodies were assayed by this technique, the curves obtained could be superimposed on the Tg standard curve, showing that serum constituents do not interfere with the assay (Figure 2). Similar results were obtained with three other serum samples.

![Fig. 1. Standard curves for human thyroglobulin, as assayed with the present technique](image1)

(a), 0–1000 μg/L; (b), 0–125 μg/L

**Fig. 2. Lack of interference of serum factors on the assay of Tg:** (x), serial dilutions of a serum containing a high initial concentration of Tg; (●), Tg standard solutions

To study the effect of autoantibodies on our IRMA-Tg procedure, we determined the concentration of Tg in undiluted sera containing elevated (>8000 μg/L) concentrations of anti-Tg aAbs to which different Tg standard solutions were added. The results of this recovery experiment (Figure 3) show that within the concentration range of 0 to 100 μg of added Tg per liter, this curve paralleled the standard curve, indicating that there was no interference by the aAbs. The difference between the curves for the test serum and the standard Tg solution represents the initial Tg concentration in the serum. When the concentration of added Tg was >100 μg/L, the high concentration of aAbs slightly interfered with the assay. Low concentrations of anti-Tg aAbs (≥1500 μg/L) did not interfere with the assay over the entire concentration range (results not shown).

The intra-assay coefficient of variation (six sera) and the interassay coefficient (five sera) were <7% from 30 to 800 μg/L.

We compared the Tg concentrations in 40 sera as determined by the present technique with those estimated by use of a commercial IRMA kit (SB-HTG; ORIS Industrie). Of these sera, 17 contained anti-Tg aAbs (absorbance >1.0 at 405 nm for serum diluted 10-fold when the indirect enzyme-linked immunosorbent assay technique was used). The coefficients of correlation between the two techniques were 0.98 for sera containing aAbs and 0.96 for sera that did not contain aAbs (Figure 4). The correlation between the two methods is significant in both cases (P <0.001).

It is important to note that the concentration of Tg in

![Fig. 3. Assay of Tg in serum containing 8000 μg of aAbs per liter (△) and standard curve (●)](image2)
serum determined by our technique exceeded that measured with the commercial IRMA kit, particularly when Tg concentrations were <100 µg/L as measured by the present procedure. The slopes of the regression lines for the two populations of sera (Tg concentrations <100 µg/L and >100 µg/L) were 0.55 and 0.86, respectively.

Discussion

In a previous study (12), we showed that the anti-Tg aAbs in the serum of patients with thyroid disorders recognize mainly two antigenic regions (II and IV) on the human Tg molecule among the six recognized by our battery of 15 anti-Tg MABs. For the development of the Magnogel-IRMA-Tg technique, we chose those 10 anti-Tg MABs in our collection that do not recognize these two regions.

In preliminary experiments with these MABs, we tried to assay Tg in serum by an immunoenzymometric assay, using only one of the MABs to trap the Tg molecule and another one as the second antibody. All combinations of the 10 MABs (recognizing regions I, III, V, VI) were tested but without success. These results suggested to us that there might be several different molecular forms of Tg in the serum. Some of them might be the result of Tg fragmentation. If so, it should be possible to capture the fragments by using a combination of MABs coupled to the magnetic microbeads (Magnogel). This hypothesis appeared to be correct because our results showed that we were able to assay Tg in serum by using four MABs to trap the antigen and one MAB as the second antibody. This latter MAB appears to behave as a universal probe for the different forms of Tg, and it is probably directed against a very stable epitope.

The IRMA-Tg technique reported here is reproducible, rapid (5 h), and sensitive (lower limit of detection: 3 µg/L), and serum constituents do not appear to interfere. Furthermore, the technique can be used when the test serum contains anti-Tg aAbs. However, we noted that the presence of these aAbs at a very high concentration leads to a slight underestimation of Tg in serum when the concentration exceeds 100 µg/L. It should be underscored that a Tg concentration in serum >100 µg/L is indicative of a thyroid disorder (7) and, as such, the minor interference observed in this case should not have significant clinical consequences.

The sensitivity of our technique makes this assay valuable for monitoring circulating Tg in patients with thyroid carcinoma who have undergone total ablation of the thyroid gland, even when aAbs are present in their serum.

This new method based on a panel of MABs directed against several antigenic determinants on the Tg molecule that are not recognized by anti-Tg aAbs can be used to assay Tg in serum of patients with various thyroid pathologies.

We thank Dr. H. Borel for providing us with serum samples, Ma. V. Mauries for excellent technical assistance, and Dr. S. L. Salhi for critical comments and editorial assistance. This work was supported by Sandofi-Recherche, to whom we extend our gratitude.

References