Development and Utility of a Monoclonal-antibody-Based, Highly Sensitive Immunoradiometric Assay of Thyrotropin

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Using improved selection techniques, we isolated four monoclonal antibodies with high affinity for human thyrotropin (KA = 1.6 × 1010 to 2.6 × 1010 L/mol). We used two of these in an immunoradiometric assay (IRMA) that also incorporates a novel phase-separation technology (Sucrose TSH IRMA, Boots-Celltech). This assay's very low detection limit for TSH (0.03–0.08 milli-int. unit/L) and wide working range (0–250 milli-int. unit/L) allow the differential diagnosis of hyperthyroid, euthyroid, and hypothyroid patients. We compare the utility of this IRMA with that of a RIA for patients with various thyroid disorders. As determined by IRMA, a normal concentration of TSH in serum excludes hyperthyroidism or hypothyroidism, whereas an undetectable serum TSH concentration (<0.08 milli-int. unit/L) accurately predicts an abnormality in thyroid gland function.

Additional Keyphrases: thyroid function · RIA compared

In highly sensitive radioimmunoassays (RIA) for thyrotropin (TSH) in serum (e.g., in 1, 2) polyclonal antisera and classical, competitive binding reactions are used.8 Time-consuming to perform, they often require incubation for several days or sample extraction (3). Furthermore, their limited analytical sensitivity does not permit one to distinguish clearly between basal TSH values, for serum from euthyroid subjects, and the suppressed values seen in hyperthyroid patients.

The development of monoclonal antibodies was expected to remove these limitations, and one of us (E.C.R.) has previously described a highly specific monoclonal antibody to human TSH (4). Although this antibody had markedly improved specificity for TSH and was well-suited to immunofluorescence studies, its affinity was lower than that of the corresponding polyclonal antisera, and it thus did not improve the performance of the resulting RIA.

Fortunately, there have been further advances in monoclonal antibody technology and immunossay technique. Here we describe the production and selection of several monoclonal antibodies to TSH and their use in development of a highly sensitive two-site immunoradiometric assay (IRMA). We also compare the analytical and clinical performance of this IRMA with that of a RIA for TSH that one of us previously developed (2).

Materials and Methods

Monoclonal Antibodies

Immunizations: BALB/c female mice were immunized with purified human TSH (TSH 10; DHSS/MRC Committee on Pituitary Hormones, U.K.). Mice received an initial subcutaneous dose of TSH in Freund's complete adjuvant (Gibco Ltd., Paisley, U.K.); a second dose by the same route, in Freund's incomplete adjuvant (Gibco); and a final dose, intravenously, in phosphate-buffered isotonic saline (PBS), three days before the spleen was removed for fusion. TSH dose regimens were either (a) 25 μg (time zero), 1 μg (week 25), and 10 μg (week 31) or (b) 1 μg (time zero), 1 μg (week 20), and 10 μg (week 26). Circulating antibodies to TSH were demonstrated by RIA before the final dose was given.

Cell fusion: Spleens were removed and the fusions carried out as previously described (5), with use of a mouse myeloma parent cell line, P3-NS1/Ag4 1 (6) and 350 g of polyethylene glycol 1540/L (Koch Light Ltd., Harverhill, U.K.) as fusogen.

Screening immunoassay: Monoclonal antibodies were detected by liquid-phase RIA. We collected culture supernates (100 μL) from near-confluent cells. Undiluted and 20-fold-diluted supernates (100 μL) were incubated with 125I-labeled TSH (4 ng/ml) for 16 h at 25°C. To separate antibody-bound 125I-labeled TSH from unbound ligand, we used a particular solid-phase-bound second antibody (sheep anti-mouse immunoglobulin, Scottish Antibody Production Unit, Carluke, Scotland) covalently linked to Sepharclyl S-300 (Pharmacia, Uppsala, Sweden) and a novel sucrose-layering separation method that does not involve centrifugation, described elsewhere (7). Radioactivity was counted in an LKB 1260 multi-well gamma counter. Hybridomas secreting high-affinity TSH antibodies, that showed equally high binding of 125I-labeled TSH whether they were undiluted or diluted 20-fold, were selected, stabilized by subcloning, and stored in liquid nitrogen.

Antibody specificity: We determined this, using the screening RIA described above but incubating the antibodies with 125I-labeled lutropin or 125I-labeled follitropin. Only hybridoma cells secreting TSH-specific antibodies were investigated further.

Affinity estimates: We estimated relative binding affinities for TSH by determining the binding of 125I-labeled TSH at various dilutions of each culture supernate, as described previously by one of us (8). Affinity constants (KA) were estimated by the method of Adrion (9) from the antibody dilution giving 50% maximal binding of 125I-labeled TSH.

Antibody selection: We further evaluated the four highest-affinity TSH-specific monoclonal antibodies ("24," "258," and "296" from immunization procedure a and "323" from procedure b) for their potential use in a two-site IRMA. After subcloning, asitic fluid was grown from each hybridoma in pristine-treated BALB/c mice. Antibodies, separated from asitic fluid by precipitation with 180 g/L sodium sulfate

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5 Nonstandard abbreviations: TSH, thyropitropin; IRMA, immunoradiometric assay; PBS, phosphate-buffered isotonic saline; T4, thyroxin; T3, triiodothyronine.
6 Received December 27, 1986; accepted May 22, 1987.
solution, were used to prepare solid-phase and radiolabeled antibody preparations. We coupled sodium sulfate fractions from each monoclonal antibody and from a sheep polyclonal anti-TSH serum (APU) to periodate-activated Sepharacyl-S300 (Pharmacia) as previously described (10). After further purifying each fraction by gel-filtration on Sephadex G200 (Pharmacia) we also prepared radiolabeled monoclonal antibody with Na125I (Amersham PLC, Amersham, U.K.), using a Chloramine-T procedure (11). We then sought complementary pairs by studying each possible combination of solid-phase and radiolabeled antibody in a two-site IRMA.

Final purification of selected monoclonal antibodies: Monoclonal antibody 323, selected for the IRMA radiolabel, was produced in 100-L tissue culture in an air-lift fermenter (12) and purified from culture fluid by affinity chromatography on Protein A-Sepharose (Pharmacia) followed by gel-filtration chromatography on Sephacryl S-300. Monoclonal antibody 42, used for the IRMA solid phase, was purified from ascitic fluid by preparative ion-exchange chromatography.

Immunooassays

Two-site IRMA: In this assay (Sucrosep TSH IRMA, Boots-Celltech Diagnostics) either 100 μL of TSH standard (human pituitary TSH, Porton, U.K.) in standard diluent (350 mL of bovine serum per liter of 0.25 mol/L Tris buffer, pH 8.5) or 100 μL of serum sample is incubated with 100 μL of 125I-labeled monoclonal antibody 323 (~30 ng/mL; specific activity 450–600 KBq/μg) for 2 h at room temperature (15–30 °C) in 12 × 75 mm plastic tubes. One hundred microliters of a suspension (10% by vol) of solid-phase antibody 42 in the Tris buffer is then added and the tubes are shaken on an orbital agitator for a further hour. Free and solid-phase-bound 125I-labeled monoclonal antibody fractions are separated by the sucrose-separation technique (7) and the radioactivity of the bound fraction is counted for 1 min in a gamma-scintillation counter having a background count rate less than ~60 counts/min. In this study, we processed immunooassay data by using a five-parameter log-logistic curve fit (Boots-Celltech Diagnostics Ltd.) and an IBM-PC microcomputer.

Radioimmunooassays (RIA): The TSH RIA has been described previously (4). Briefly, in the procedure a rabbit polyclonal antiserum to human TSH is used and the total incubation time is seven days. This RIA has a formal detection limit of 0.3–0.5 milli-int. units/L. Thyroxin (T₄), triiodothyronine (T₃), and T₂-resin uptake assays were performed as previously described (13).

Patients

Serum was sampled from patients referred to the Thyroid Clinic of the Massachusetts General Hospital, Boston, MA. Unless stated, these patients were not taking thyroid medication and had no history of hypothalamic–pituitary disease. We performed routine thyroid-function tests on all specimens, including determinations of serum T₃ and T₄ and T₂-resin uptake. We measured serum TSH by both RIA and IRMA.

On the basis of clinical history, physical examination, and results of routine thyroid-function tests we classified 65 subjects as euthyroid. Of these, 23 were normal volunteers who also had normal TSH responses by RIA to thyrotropin stimulation (2). Twenty-six subjects had classical hypothyroidism and 31 primary hypothyroidism. We also studied 58 patients with primary hypothyroidism who were being treated with various dosages of L-thyroxin (75–250 μg/d).

Fifteen subjects had autonomously functioning thyroid nodules, as defined by "toxic" hyperfunctioning nodules, because values for either T₄ or T₃ in their serum were abnormally high. Twenty-one patients had characteristic multinodular goiters with normal values for T₄, T₂, and T₃ resin uptake; none had clinical symptoms or signs of hyperthyroidism.

Results

Characterization of monoclonal antibodies: Calculated Kₜ values ranged from 1.6 × 10⁶ to 2.6 × 10¹⁰ L per mole of TSH. We found that the slope and position of the IRMA dose-response curve for each 125I-labeled antibody used in combination with the solid-phase polyclonal anti-TSH serum was related to the Kₜ value calculated from RIA data. We also studied complementation of the four antibodies in the two-site IRMA. The non-complementary nature of antibodies 42 and 258 suggested that these antibodies recognize the same (or nearly adjacent) epitopes on the TSH molecule; all other combinations were permissible. We selected the two antibodies of highest affinity (antibody 323, Kₜ = 9.6 × 10⁶ L/mol; antibody 42, Kₜ = 2.6 × 10¹² L/mol) for the Sucrosep IRMA.

Optimization of IRMA: Although antibody 42 had the higher estimated Kₜ value, we found that the TSH dose–response curve of the IRMA was most sensitive when we used 125I-labeled antibody 323 and solid-phase antibody 42. We improved assay sensitivity by delaying the addition of the solid-phase reagent. As expected, we found the percentage of 125I-labeled monoclonal antibody bound in the IRMA to be related to its mass and we selected a concentration of 3.0 ng per tube for this reagent, a compromise between the requirements for TSH detection limit, working range, and acceptable count-rate. We selected solid-phase antibody concentrations in a similar series of experiments so as to ensure that this reagent was in true molar excess relative to TSH. In a series of quantitative recovery experiments, in which we used TSH (International Reference Preparation 68/38) added to 20 individual normal sera, we demonstrated the validity of using 350 mL of bovine serum per liter of Tris buffer as a matrix for the assay standards. Mean analytical recoveries of added TSH (0.54–57.1 milli-int. units/L) ranged between 95.6% (SD 3.4%) and 104.8% (SD 5.3%).

Analytical performance of the IRMA: Figure 1 depicts a typical standard calibration curve for the IRMA and an associated within-assay precision profile. Typical assay sen-
sitivity (detection limit), defined as the smallest concentration of TSH that differs significantly from the zero standard (±2.5 SD), is 0.03–0.08 milli-int. unit/L. The range within which the assay has a within-assay CV <10% is 0.4–250 milli-int. units/L.

The specificity of the assay was determined in two ways (Table 1): by assessing the cross reactivity of related hormones in the absence of TSH and by measuring interference by substances in the presence of TSH. We attribute the interference (<15%) by extremely high concentrations of human chorionic gonadotropin to low-affinity recognition of this hormone by the 125I-labeled monoclonal antibody (no. 323). This interfering substance is not recognized by the solid-phase antibody and does not cross react in the IRMA.

Clinical performance of the IRMA: We compared results for TSH obtained with the IRMA with those by RIA (Table 2).

Eight of the 65 (12%) euthyroid subjects had undetectable TSH in the RIA, but TSH was detectable in all samples by the more-sensitive IRMA. Results for samples giving TSH values within the normal range for the RIA agreed well with TSH values measured by IRMA (r = 0.83, p <0.001).

For the 23 normal volunteer subjects given stimulation with thyroliberin (200 µg, intravenously) the mean peak TSH response was 10.8 (2SD = 4.8) milli-int. units/L by RIA and 16.0 (2SD =11.0) milli-int. units/L by IRMA.

All eight euthyroid subjects who had undetectable basal TSH by RIA subsequently had normal TSH responses to thyroliberin.

Hyperthyroid patients had undetectable TSH values by both RIA (<0.5 milli-int. unit/L) and IRMA (<0.08 milli-int. unit/L) in both basal samples and samples collected after thyroliberin injection.

The wider working range of the IRMA allowed us to assay all but one of the hyperthyroid patients’ sera without dilution, whereas all required dilution before analysis by RIA.

The IRMA results for hypothyroid samples by the two methods correlated well (r = 0.88, p <0.001). In hypothyroid patients treated with L-thyroxin (75 to 250 µg daily) we found the basal value for TSH in serum by IRMA more accurately predicted incomplete pituitary suppression; TSH was detectable in the serum of only 19 of 58 (33%) patients by RIA but in 35 of the 58 (61%) by IRMA.

All of the 15 patients who had a solitary hyperfunctioning thyroid nodule had undetectable basal serum TSH (<0.5 milli-int. unit/L) in the RIA and low TSH values (<0.08–0.23 milli-int. unit/L) in the IRMA. Similarly, we found that patients with multinodular goiters who were euthyroid had serum TSH values extending from the undetectable range to the normal range in both the RIA and the IRMA. However, by RIA, 11 of the 21 patients (52%) had undetectable TSH values, whereas by IRMA only one (4%) of these patients had undetectable serum TSH.

Discussion

Until recently, the clinical utility of serum TSH determinations has generally been limited to that of an ancillary test in the confirmation of hypothyroid status after a primary finding of low thyroid-hormone concentrations in serum. Because of this limited analytical sensitivity, TSH RIA has not been helpful in the diagnosis of hyperthyroidism because the lower limit of the normal range for TSH is undefined.

Our previous isolation of a specific anti-TSH monoclonal antibody (4) failed to improve assay sensitivity significantly. We attributed this to the antibody’s relatively low affinity for TSH (K = 4.0 × 10⁶ L/mol) and to certain deficiencies in the techniques then available for monoclonal antibody selection and immunoassay.

In this study, we demonstrate that it is possible to improve TSH measurement substantially by using monoclonal antibodies with affinities that are equal to or even lower than those found in many polyclonal antisera, if certain improvements in antibody selection and immunoassay design are used. For example, our use of antibody-dilution curves for 125I-hormone preparations allowed us to select high-affinity antibodies at a very early stage after cell fusion.

The assay described involves a novel separation technique (Sucrosep) to achieve the full dose–response potential of the two-site IRMA. This technique provides very efficient washing of the solid-phase and results in excellent separation of bound and free radioactive fractions without centrifugation. After two washes, fewer than 50 counts per minute (from a total >50 000) of 125I-radiolabeled antibody are associated with the solid-phase in the absence of TSH: count misclassification rate of <0.1%. The resulting assay (Figure 1) is characterized by high sensitivity, excellent within-batch precision, and a working range that covers the entire pathophysiologial range for TSH, a working range that obviates diluting sera from hypothyroid patients before

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**Table 1. Cross Reactivity and Interference by Related Glycoprotein Hormones in TSH IRMA**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cross-reactivity studies*</th>
<th>Cross-reaction, %b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine TSH</td>
<td>100</td>
<td>c</td>
</tr>
<tr>
<td>Somatotropin</td>
<td>0.3</td>
<td>c</td>
</tr>
<tr>
<td>Prolactin</td>
<td>2.3</td>
<td>c</td>
</tr>
<tr>
<td>Lutropin</td>
<td>60</td>
<td>0.0005</td>
</tr>
<tr>
<td>Follicropin</td>
<td>0.3</td>
<td>0.0002</td>
</tr>
<tr>
<td>Chorionadotropin</td>
<td>50,000</td>
<td>0</td>
</tr>
<tr>
<td>*Initial TSH concn, zero.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interference studiesd</td>
<td>60</td>
<td>3.94</td>
</tr>
<tr>
<td>Lutropin</td>
<td>32</td>
<td>3.84</td>
</tr>
<tr>
<td>Follicropin</td>
<td>32</td>
<td>3.41</td>
</tr>
<tr>
<td>Chorionadotropin</td>
<td>50,000</td>
<td>13.2</td>
</tr>
</tbody>
</table>

*Initial TSH concn, zero.  
b(Measured TSH concn/cros-reactant concn) × 100.  
c(NDetectable (<0.06 milli-int. unit/L).  
dInitial TSH concn, 3.93 milli-int. units/L.  
*Decrease in measured TSH concn/initial TSH concn) × 100.

**Table 2. Basal TSH Concentrations in Sera from Patients with Thyroid Disorders: RIA and IRMA Compared**

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>No. of patients</th>
<th>RIA, milli-int. units/L</th>
<th>IRMA, milli-int. units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>65</td>
<td>&lt;0.5–3.50</td>
<td>0.40–5.0</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>26</td>
<td>&lt;0.5</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>31</td>
<td>10–513</td>
<td>17–417</td>
</tr>
<tr>
<td>Hyperfunctioning thyroid nodule</td>
<td>15</td>
<td>&lt;0.5</td>
<td>&lt;0.08–0.23</td>
</tr>
<tr>
<td>Multinodular goiter</td>
<td>21</td>
<td>&lt;0.5–1.70</td>
<td>&lt;0.08–2.20</td>
</tr>
</tbody>
</table>

*Mean and SD.*
This monoclonal-antibody-based IRMA also exhibits excellent specificity. In any two-site assay, we believe it is important to distinguish between the situation where related substances cross react (i.e., are recognized by both of the pair of monoclonal antibodies, giving rise to analytical overestimation) and those where substances interfere (i.e., are recognized by only a single member of the pair, giving rise to analytical underestimation). Unlike many RIAs, this TSH IRMA shows no significant cross reaction with related hormones, including chorionic gonadotropin. Although it does show slight interference by extremely high concentrations of hCG, we believe this is unlikely to be of diagnostic significance. In fact, this IRMA has recently allowed small, previously unrecognized physiological declines in TSH during early pregnancy to be identified (14).

We, and others (15–19), have shown that these analytical advantages of the IRMA extend the clinical utility of TSH assay. In contrast to RIA, the increased analytical sensitivity of the IRMA allowed TSH to be detected in basal sera from all the euthyroid subjects studied; if a basal value for TSH in serum by IRMA falls within the normal range (0.5–5.0 milli-int. units/L), it excludes a diagnosis of either hyperthyroidism or hypothyroidism. Similarly, an undetectable TSH by IRMA (but not by RIA) predicts an abnormality of thyroid function and is consistent with a number of disorders, including hyperthyroidism, hyperfunctioning thyroid nodule, multinodular goiter, and thyroid hormone suppression. In this instance, additional tests are required for a precise diagnosis. The IRMA’s ability to discriminate completely between hyperthyroid, euthyroid, and hypothryroid subjects confirms the suggestion (15, 16) that basal serum TSH determination by IRMA may suffice in the initial investigation of patients referred for thyroid evaluation. This allows thyroid testing strategies to be rationalized, and may decrease the total number of tests required (19).

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References