Two-Monoclonal- Antibody Sandwich-Type Assay for Thyrotropin, With Use of an Avidin–Biotin Separation Technique

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We have developed a sensitive, specific, noncompetitive, sandwich-type radioimmunoassay for human thyrotropin (hTSH), which can be performed in 30 min. The assay involves two monoclonal antibodies, selected for high affinity and specificity and also for reaction against antigenic sites on hTSH that are distal from each other. One of these antibodies is labeled with 125I; the other is conjugated covalently to biotin. Polystyrene beads were also conjugated covalently to biotin. After conjugation, the beads were incubated with avidin. These beads represent a rapid, simple method for separating hTSH-bound antibody from free antibody. The biotin–antibody–hTSH–125I-labeled antibody complexes bind to the beads and hTSH concentration is directly related to counts per minute. This assay can detect hTSH at a concentration of 0.06 milli-unit/L in serum.

The first radioimmunoassays permitting measurement of human thyrotropin (hTSH) in serum were described by Utiger, Odell, and coworkers (1–3). These assays represented a significant advance, were used for physiological studies (4), and reliably distinguished serum of euthyroid subjects from that of subjects with primary hypothyroidism. They were not sensitive enough reproducibly to distinguish hyperthyroidism from normal, or for use in studying the dynamics of hTSH secretion (e.g., diurnal variations) in normal subjects. Subsequently, assays of increasing sensitivity have been described that can be used for such purposes, but they have required either extraction of serum (5) or five- to seven-day incubations and very low antibody-bound/free (B/F) hormone ratios (6–8). These assays have been called "competitive assays" because the hTSH in the sample competes with radiiodinated hTSH for a limited number of antibody binding sites. In 1968, Miles and Hales (9, 10) described the "excess" reagent principle for radioimmunoassay, which they termed the "immunoradiometric assay system." At that time only polyclonal antibodies were available, which limited specificity and ability to select specific hormonal antigenic sites. Their technique was applied to hTSH by Seth et al. (11), who used purified polyclonal antibodies in demonstrating the striking increase in sensitivity that was possible. With the advent of techniques for production of monoclonal antibodies (12, 13), an additional principle could be added: production and selection of two or more antibodies directed against spatially distant sites on a single hormone or analyte. By use of such antibodies in highly purified form, so-called "excess reagent," nonequilibrium assays have been developed (14, 15). Such assays have the advantages of much greater sensitivity and very short incubations as compared with competitive assays.

We have developed such an assay for hTSH, which can be performed in 30 min. Herein we describe our method for development and use of the assay system. We have prepared the first detailed dose–response curves for in vivo immunization of mice with hTSH, data that permit selecting the optimal yet minimal dose for monoclonal antibody production. In addition, we describe methods for screening antibodies for reactivity with spatially distant antigenic sites on a hapten or analyte molecule; we also describe the use of the avidin–biotin reaction as a separation method for sandwich assays (16, 17).

Materials and Methods

Antibody Production

Six groups, each consisting of six or seven Swiss Websters (Simonsen, CA) female mice, were immunized with highly purified hTSH (NIADDK-hTSH-F-5) (AFP-4370B) in various doses. Each dose of hTSH was diluted in 100 μL of isotonic saline, homogenized with equal volumes of complete Freund's adjuvant, and injected subcutaneously in multiple sites. Every two weeks each group of mice received one of the following hTSH immunizing doses: 0.1, 1.0, 3.0, 10, or 30 μg. Just before the third and all subsequent immunizations, blood was sampled from the tail vein of each mouse. Using this serum as a source of polyclonal anti-hTSH, we incubated the antibodies with radiiodinated hTSH to determine the titer of the antisera. Antibody-bound hTSH was determined by precipitation with antiserum to mouse gammaglobulin in a conventional "double-antibody" assay system.

Based on titer response, mice that produced the highest antibody titer were chosen for fusion. Three days before the fusion, 10 μg of hTSH in 200 to 300 μL of isotonic saline was administered as a booster dose via the tail vein.

Fusion was done according to usual procedures; we followed a procedure described by Oi and Herzenberg (12). The myeloma cell line used was SP2/0 Ag 14, kindly supplied by Seth Pincus, M.D., Department of Pediatrics, University of Utah Medical Center, Salt Lake City.

Purification of Antibody

An immuno-affinity column was used to purify small amounts of antibody from culture supernates. Monoclonal rat anti-mouse kappa chain (5 mg), also supplied by Seth Pincus, was bound to 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated with Tris buffer (0.1 mol/L, pH 7.6) containing 9 g of NaCl per liter. Five to 10 mL of supernate was
applied to the top of the column and eluted with a 3.5 mol/L solution of magnesium chloride; 1-mL fractions were collected. A spectrophotometer was used to determine which fractions contained the antibody and the concentration of antibody (absorbance of 1.46 at 280 nm corresponds to 1 mg of IgG per milliliter). The fraction or fractions were pooled and dialyzed against phosphate-buffered saline (per liter, 10 mmol of sodium phosphate and 150 mmol of sodium chloride, pH 7.4). Antibody production in supernatant fluids ranged from 10 to 60 μg/mL. After purification, 30 μg of each purified antibody was iodinated with the use of Chloramine T, as previously described (19).

Ascites fluid was produced as follows, to make larger quantities of antibody. Nude mice were injected intraperitoneally with 0.5 mL of pristane 10 days and three days before injection with live cells (2 × 10⁶ cells per animal). Within a week the animals were killed and the ascites fluid was harvested. Antibody was precipitated with 30 and 50 g of ammonium sulfate per 100 mL. The precipitates were redissolved in phosphate-buffered saline at one-tenth of the original volume. This was applied to a column of Sephadex G200 that had been equilibrated with phosphate-buffered saline to separate the lipids from the IgG and serum albumin, the latter two producing peaks that absorb at 280 nm. Those fractions containing IgG and serum albumin were pooled, dialyzed against potassium phosphate buffer (10 mmol/L, pH 6.8) and applied to a diethylaminoethyl (DEAE) Sephadex column equilibrated with the same buffer. The antibody passed through the column with the void volume and was collected; the albumin contaminants remained on the column. Fractions containing antibody were pooled and dialyzed against phosphate-buffered saline, diluted to 1 mg/mL, aliquoted, and stored frozen at −70 °C.

Iodination of Monoclonal Antibody

With some modifications, monoclonal antibody was radioiodinated by the Chloramine T method (19). Thirty microliters (30 μg) of purified monoclonal antibody was pipetted into a test tube, then mixed gently with 75 μL of 0.5 mol/L phosphate buffer (pH 7.5), 1 mCi of carrier-free ¹²⁵I, and 2.5 μg of Chloramine T in 5 μL of phosphate-buffered saline. After 1 min, the reaction was stopped by adding 5 μg of sodium metabisulfite in 10 μL of phosphate-buffered saline. The vial was rinsed twice with 300 μL of 10 g/L bovine serum albumin in phosphate buffer, and the combined mixture was transferred to a Sephadex G-75 Superfine column for purification. Fractions of 1 mL were collected in tubes containing 500 μL of antibody buffer and tested for binding activity.

Screening of Monoclonal Antibodies

We purified 10 monoclonal antibodies that had been both iodinated and biotinylated as described. Using the solid-phase assay (see below), we tested each iodinated antibody in the presence of hTSH and biotinylated antibody. All possible combinations of iodinated antibodies and biotinylated antibodies were tested (in a chi-square design). The combinations of antibodies having the higher affinities for hTSH were chosen for assay development. Of the 10 antibodies screened, two combinations produced a highly sensitive assay.

Assay Procedure

Biotinylated monoclonal antibody was diluted with the radiiodinated antibody buffer (described above) to a concentration of 0.5 μg of biotinylated antibody and 300 000 counts/min of radiolabeled antibody in a total volume of 100 μL. The assay was performed by combining 100 μL of standard or serum sample, 100 μL of antibody solution, and one avidin-coated bead in a test tube. The tubes were incubated for 2 h at room temperature on a rotating shaker at 170 rpm. The liquid was aspirated from the bead, and the bead was washed twice with 2 mL of phosphate-buffered saline containing 1 mL of Triton X-100, and its radioactivity was counted for 1 min in a gamma spectrometer.

Results

The six groups of mice produced antisera in vivo with titers that were directly related to the dose of immunogen
administered. At all times of testing, beginning after the second immunizing dose, this dose–response relationship was apparent. Figure 1 shows the average titer plotted against dose of immunogen after five injections. As little as 1 μg produced a significant titer; 10 and 30 μg produced maximal titers. The animals used for fusion were selected from the groups receiving 10- and 30-μg immunizing doses. The initial fusions were performed to develop the rapid screening method (18) and were not used in the hTSH assay development.

Cells from a single animal were used for fusion to produce the hTSH assay described here. After fusion, the initial cultures were screened for anti-hTSH production in vitro. From this fusion, 48 parent lines were produced. These parent lines all produced detectable anti-hTSH. Aliquots of all parent lines were frozen and survived. After cloning, 864 colonies grew and 154 (18%) of these produced detectable anti-hTSH. Twenty-five of these were selected for further characterization. Nine of these clones, which produced the highest titer in vitro, were selected for larger-scale antibody production and antibody purification. Table 1 summarizes these data.

The purified monoclonal antibodies were selected on the basis of: (a) the mouse that produced the highest titer of anti-hTSH in vivo and (b) the hybridoma cell lines that produced the highest titer in vitro. They were tested systematically to determine which pairs reacted against spatially distant antigenic sites on the hTSH molecule. We assumed in these experiments that when one antibody interfered with binding of a second antibody, the antigenic sites were located close together on the hTSH molecule. Table 2 shows the results of these screening studies. In addition to these antibody studies, one of us (R.Z.), working independently at Nichola Institute, screened commercially available monoclonal antibodies in a similar fashion and selected three on the basis of similar criteria for assay development. However, none of the monoclonal antibodies against hTSH discussed in this manuscript were used for assay development at Nichola Institute.

Using antibodies nos. 6, 9, 10, 15, and 21 (Table 2), we constructed hTSH dose–response curves. From these results, two antibodies (nos. 6 and 9) were selected for assay development; we then assessed the effects of time and temperature on the results of assays involving these antibodies. Figure 2 shows the data from some of these studies and indicates that longer incubations increase the radioactivity bound at all doses of hTSH; however, the sensitivity of the assay changes little after 30 min. For routine use in our laboratory, we selected a 2-h incubation, shorter times being almost too rapid for our practical use; we emphasize, however, that shorter incubations may be used.

Studies were also performed to assess the effects of serum components on the assay by incubating serum from patients with active Graves' disease (we assume no detectable hTSH) and comparing the counts bound with those of buffer controls. By such assessment we detected no nonspecific serum effects. However, because rheumatoid factor has been described to react with heterologous immunoglobulins (20) and could react with mouse immunoglobulins, we also assessed sera with positive rheumatoid factor from euthyroid patients with rheumatoid arthritis. Such sera produced artifically high hTSH values. To remove this possible source of spurious hTSH values, we added normal mouse immunoglobulin to the assay buffer as normal mouse serum, 2.5 mL/L. Under these conditions, rheumatoid factor-posi-

Table 1. Outline of Anti-hTSH Monoclonal Antibody Development

<table>
<thead>
<tr>
<th>Activity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>38 mice immunized</td>
<td></td>
</tr>
<tr>
<td>1 fusion</td>
<td></td>
</tr>
<tr>
<td>48 parent lines in 2-mL wells—</td>
<td>100% produced anti-hTSH</td>
</tr>
<tr>
<td>25 cloned</td>
<td></td>
</tr>
<tr>
<td>864 colonies grew</td>
<td></td>
</tr>
<tr>
<td>154 (18%) produced anti-hTSH</td>
<td></td>
</tr>
<tr>
<td>25 clones selected for antibody</td>
<td>characterization</td>
</tr>
<tr>
<td>10 antibodies selected for purification/characterization</td>
<td></td>
</tr>
<tr>
<td>2 antibodies selected for assay development</td>
<td></td>
</tr>
</tbody>
</table>

![Graph](image)

**Fig. 1. Anti-hTSH titer after five immunizations with hTSH two weeks apart**

Titer was assayed as antiserum dilution that binds 50% of 125I-labeled hTSH under equilibrium double-antibody assay conditions. Each point represents antiserum from one animal (38 mice total); the horizontal lines show the mean titer at each immunization dose

*With reference to the international units defined for the 2nd IRP hTSH (no. 80/658) from the World Health Organization.
Table 2. Screening for Monoclonal Antibodies that Recognize Antigenic Sites Located Distantly from Each Other

<table>
<thead>
<tr>
<th>Radioiodinated antibody, no.</th>
<th>4</th>
<th>6</th>
<th>9</th>
<th>10</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>17</th>
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<th>21</th>
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<td>4</td>
<td>737*</td>
<td>0</td>
<td>660</td>
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<tr>
<td>6</td>
<td>429</td>
<td>375</td>
<td>372</td>
<td>411</td>
<td>259</td>
<td>267</td>
<td>392</td>
<td>133</td>
<td>254</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>3192</td>
<td>2741</td>
<td>0</td>
<td>0</td>
<td>1107</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1651</td>
</tr>
<tr>
<td>10</td>
<td>451</td>
<td>662</td>
<td>550</td>
<td>510</td>
<td>312</td>
<td>293</td>
<td>535</td>
<td>187</td>
<td>0</td>
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<tr>
<td>13</td>
<td>0</td>
<td>821</td>
<td>865</td>
<td>0</td>
<td>0</td>
<td>398</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>177</td>
</tr>
<tr>
<td>15</td>
<td>374</td>
<td>553</td>
<td>385</td>
<td>1423</td>
<td>424</td>
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<td>332</td>
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<td>17</td>
<td>0</td>
<td>3388</td>
<td>3114</td>
<td>0</td>
<td>0</td>
<td>1814</td>
<td>0</td>
<td>0</td>
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<td>2014</td>
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<tr>
<td>18</td>
<td>0</td>
<td>1430</td>
<td>965</td>
<td>0</td>
<td>0</td>
<td>543</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>551</td>
</tr>
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</table>

* % bound at dose of 600 pg of hTSH. Note that many antibody combinations (e.g., no. 9 & 4, or no. 13 & 4) compete for binding and no radioiodinated antibody is observed (counts/min = 0). Other combinations (e.g., no. 8 & 9, 6 & 17) give high binding.

Fig. 2. The effect of incubation time on the hTSH assay
All reagents were added simultaneously to the assay tube. The avidin-coated bead was removed at the end of incubation, washed, and its radioactivity determined (counts/min)

Fig. 3. The "hook effect": doses of hTSH >600 milli-int. units/L show decreasing bound radioactivity

binding globulin. The TSH concentrations ranged from 0.7 to 4.1 milli-int. units/L and were not normally distributed when plotted on an arithmetic scale (Figure 4). Such values appeared normally distributed on a log scale: 1.7 milli-int. units/L, 95% confidence limits 0.74 to 3.9 milli-int. units/L.

We studied 65 samples from thyrotoxic subjects. The subjects had also been defined independently of hTSH concentrations by their above-normal concentrations of serum thyroxin and (or) triiodothyronine (above-normal triiodothyronine resin uptake values). All but four of these patients had undetectable hTSH concentrations (<0.1 milli-int. unit/L). The four with detectable concentrations had 0.19, 0.24, 0.30, and 0.38 milli-int. unit of hTSH per liter.

Fig. 4. Histogram of the normal hTSH concentration (range 0.7 to 4.5 milli-int. units/L)
Transformed to a log scale, they were normally distributed; the mean value was 1.7 milli-int. units/L and the 95% confidence limits were 0.74 to 3.9 milli-int. units/L.

We also studied 41 samples from patients with primary hypothyroidism—low serum thyroxin and low triiodothyronine resin uptake values; their hTSH concentrations ranged from 10 to 290 milli-int. units/L. Figure 5 shows dose-response curves for samples from two euthyroid and three

Fig. 5. Dose-response curves for two sera from three euthyroid subjects and two hypothyroid subjects
Note that samples from euthyroid can be run at several dilutions

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hypothyroid subjects. Figure 6 shows these data from the populations of normal, hyperthyroid, and hypothyroid subjects.

The specificity of the hTSH assay system was assessed by incubating with immunochemical-grade human follitropin (2 NIAMDD), human lutropin (AFP-6332-B), and human chorionicadotropin (CR121), in amounts up to 1 µg per assay tube. The respective cross reactivities were as follows: 0.3%, 1%, and nondetectable (<0.1%). We do not know whether the reaction with lutropin and follitropin is caused by contamination of these preparations with hTSH or whether this is true cross-reaction in the assay.

The reliability of the assays was determined by repeated determination of two control sera in 10 completely independent assays (different iodination, different polystyrene bead preparations, different standard preparation, etc.) The average intra-assay CV was 3.5%, the average interassay CV 9%. The average CV for determinations of hTSH concentration <1 milli-int. units/L was 6.3%.

Discussion

Commonly, monoclonal antibodies have relatively low affinity. We hypothesized that this resulted from selection processes that are based on the large doses of immunogen usually administered as a single injection (12, 13). In the early years of immunonassay, large doses—milligrams—of immunogen were used to raise polyclonal antibodies in rabbits. In 1967, we published dose–response data for immunizing doses of purified human follicle-stimulating hormone (follitropin) and for the titers of anti-follitropin antiserum raised in rabbits (22). We found that much smaller doses of immunogen than had been previously produced maximal titers of antisera in rabbits and permitted use of smaller amounts of scarce and expensive immunogen. On the basis of this experience, we decided similarly to assess immunization doses for mice, believing that if we could optimize in vivo antisera characteristics, we would be more likely to obtain high-affinity antibodies from cells obtained from such animals in vitro. As far as we know, the dose–response data presented here are the only ones available for mice and our previous studies the only data available for rabbits (22). Not only does 10–30 µg of purified hTSH produce a maximal titer response in mice, but also, by using such hyperimmunized mice, we can obtain a relatively high percentage of cell lines (derived from the selected mouse) that produce antisera. We now have used this selection method to produce monoclonal antibodies against several other protein hormones with equal success.

The radioimmunoassay and competitive protein-binding assays described respectively and originally by Yalow and Berson (23, 24) and by Ekins (25) were so-called “competitive” assays. In such assays a limiting number of binding sites compete for binding of a radioactive “tracer” and the analyte to be measured. In such assays, the analyte being measured is distributed at equilibrium between a bound and free state. Such equilibrium assays have greatly increased sensitivity, precision, and ease of performance in comparison with bioassays, but are highly dependent on affinity of the antibody; when optimally constructed, their limits of sensitivity for detecting protein/peptide hormones concentrations are about 10^{-11} mol/L. Given antibodies of equal affinity, the sandwich assay technique would theoretically have greater sensitivity (9, 10), almost all of the analyte reacting with antibody. In addition, sandwich assays reach equilibrium more rapidly because high concentrations of antibodies can be used. The assay we describe has a detection limit for hTSH of about 10^{-10} mol/L and can be performed in 30 min.

The avidin–biotin method of separating bound from free labeled antibody can be used for any assay involving two or more monoclonal antibodies. We prepare large quantities of avidin-coated beads and use them in several protein "sandwich" assays. It is a simple procedure and appears very reliable.

Currently, an increasing number of causes of euthyroid hyperthyroxinemia are being described (26), and it is often difficult to distinguish these patients from those with thyrotoxicosis. With this very sensitive hTSH assay we could separate completely a population of subjects with primary hyperthyroidism from normal subjects. In addition, diagnosis of patients with thyrotoxicosis and inappropriately "normal" hTSH caused by a pituitary TSH-producing tumor, or by a defect in thyronine regulation of hTSH secretion, should be possible. Finally, such assays should be useful in optimizing thyroid hormone dosage for treatment of hypothyroidism.

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