Heterophilic Antibodies Remain a Problem for the Immunoassay Laboratory

GREG WARD, MSc,1 LETITIA MCKINNON,1 TONY BADRICK, FAACB,2 AND PETER E. HICKMAN, FRCPA1

The prevalence of heterophilic antibody interference in a modern immunocheluminometric assay containing blocking agents was determined using thyrotropin as an illustrative example. Serum samples were obtained from 295 consecutive patients who underwent routine thyroid function testing. The following versions of the thyrotropin assay were used: protocol A (zero blocker), protocol B (routine blocker concentration), and protocol C (extra blocker).

Ten patients (prevalence 3.4%) had significant levels of heterophilic antibodies (protocol A value greater than 9 SD from the protocol B value). The observed thyrotropin levels for protocols B and C were the same for all patients, consistent with the reagent blockers in routine assays adequately eliminating heterophilic antibody interference. However, seven more patients (0.03%) in a series of 21,000 assessed by routine thyroid function testing had discordant results because of a concentration of heterophilic antibodies so high as to overwhelm the added blocking agents. (Key words: Heterophilic antibody; Interference; Incidence; Thyrotropin; Thyroid function) Am J Clin Pathol 1997;108:417-421.

We recently have noticed that some patients still have apparently inappropriate results, which on testing seem to be due to extremely high concentrations of heterophilic antibodies that overwhelm the added blocking agents. As a result of these observations, we decided to assess the prevalence of heterophilic antibodies in the population we serve and to try to determine the frequency of cases that can overcome blocking agents in a commercially available thyrotropin immunoassay method.

MATERIALS AND METHODS

Patient Specimens

We estimated the prevalence of heterophilic antibodies by looking at 295 consecutive routine requests for thyroid function tests. The only selection criterion was that a serum volume of 3 mL be available. Serum was stored at -20°C until required for assay.

Before this prospective study, we had identified a number of cases of major interference in thyrotropin assays by heterophilic antibodies. Each of these cases had particular points of interest, and studies on these patients’ samples are also described.

Hormone Assays

Our baseline assays for thyroid function are free T4 (thyroxine) and thyrotropin, measured on the automated
immunoassay analyzer (ACS:180, Chiron Diagnostics, Melbourne, Australia). When clinically indicated, free T₃ (triiodothyronine) was also measured using radioimmunoassay (Amerlex MAB kit, Johnson and Johnson, Melbourne, Australia).

The level of thyrotropin (second International Reference Preparation, 80/558) was measured by a two-site immunochemiluminometric assay (ICMA). This thyrotropin assay system uses immunopurified sheep and monoclonal anti-thyrotropin as the respective capture (TSH [thyroid stimulating hormone] solid-phase reagent) and labeled antibodies (TSH Lite Reagent, Chiron Diagnostics). With the routine ACS:180 TSH assay, the solid phase and Lite Reagent, respectively, contain normal sheep serum (5 mL/L reagent, 2.25 µL/reaction cuvette) and normal mouse serum (10 mL/L reagent, 1 µL/reaction cuvette) as blocking agents to adsorb endogenous heterophilic antibodies.

Three different versions of the thyrotropin assay were compared in which the concentration of blocker in the reaction cuvette was varied by reagent modification or previous addition of blocker to the sample. First, in protocol A (zero blocker), thyrotropin was measured using reagents that were identical to the routine assay, except that the added sheep and mouse serum was omitted. The routine assay (protocol B) and the assay with extra blocker (protocol C) used the reagents described in the preceding paragraph, but with protocol C, 22.22 µL of extra blocker in the reaction cuvette was achieved by incubating 50 µL of sheep serum (Sigma, Sydney, Australia) with 400 µL of patient serum before performance of the routine assay. Sheep serum was chosen because the capture antibody was raised in sheep.¹ All 295 patient samples were assayed by these three thyrotropin assays. A detailed precision profile was prepared.²

For one patient, extended studies were performed using nonimmune sera (Sigma) from several different animal species as the blocking agent.

**Statistical Methods**

The difference plot was used to compare the thyrotropin levels for each patient from each of the protocols.³ Results identified as outliers by this procedure were considered to be due to heterophilic antibody interference given the premise that as the amount of sheep serum is increased, then heterophilic antibody interference will be eliminated. The data were plotted using the graphic software Sigma Plot version 5.0 (Jandel Scientific, San Rafael, Calif).

The Kolmogoroff-Smirnoff goodness of fit test⁴ and analysis of variance (ANOVA) were used to compare data.

**RESULTS**

**Comparison of Thyrotropin Levels Observed in Protocols A and B**

A difference plot for comparison of the thyrotropin levels observed in protocols A and B was calculated (Fig). This procedure identified six patients (2%) as major outliers who had thyrotropin levels in protocol A that were at least 600% greater than those measured in protocol B (> 13 SD). The respective thyrotropin levels (mU/L) in protocols A and B for each of these patients were: 58, 2.0; 8, 0.8; 40, 1.5; 62, 5.6; 33, 3.0; and 15.0, 2.5. The results for protocol A for these six patients also would be clinical outliers because the upper limit of the reference range for thyrotropin is 5.0 mU/L. In general, the protocol A thyrotropin levels for these patients were similar to those reported for heterophilic antibody interference in thyrotropin assays in which the reagent did not contain blocking agents.⁵ Another four patients had thyrotropin levels in protocol A that were at least 150% greater than those measured in protocol B (> 9 SD).

For protocols A and B, the interassay imprecision and mean values obtained using commercial quality control material were identical (ANOVA), and for the
Heterophilic Antibodies

Baseline Thyroid Hormone Investigations in Seven Patients With Heterophilic Antibodies When First Tested

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Free ( \text{T}_4 ) (pmol/L)</th>
<th>Thyrotropin Protocol B (mU/L)</th>
<th>Thyrotropin Protocol C(mU/L)</th>
<th>Clinical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>16.5</td>
<td>0.3</td>
<td>Receiving thyroxine</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>0.07</td>
<td>&lt; 0.03</td>
<td>Thyrotoxic state</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>24.0</td>
<td>1.2</td>
<td>Possible hypothyroid</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>11.8</td>
<td>0.6</td>
<td>Receiving thyroxine</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>0.10</td>
<td>&lt; 0.03</td>
<td>Possibly thyrotoxic state</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>26.0</td>
<td>&lt; 0.03</td>
<td>Thyrotoxic state</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>3.3</td>
<td>0.30</td>
<td>OKT3 therapy</td>
</tr>
</tbody>
</table>

\( \text{T}_4 \) = thyroxine.

*Reference range, 9-23.
*Reference range, 0.3-5.0.
*The thyrotropin assay used was a modification of protocol C in which the volume of sheep serum added varied between 10 and 50 \( \mu \)L. The thyrotropin value reported is that amount at which the addition of further sheep serum did not alter the measured value.

There was no difference in the observed thyrotropin levels measured by the protocol B and protocol C assays for all 295 patient samples. Hence, no additional patients were detected in which the higher blocker concentration of protocol C was necessary to identify heterophilic antibody interference. These data are consistent with the blockers present in the protocol B assay, being sufficient to eliminate heterophilic antibody interference for this group of patients.

**Comparison of Thyrotropin Levels Observed in Protocols B and C**

For some of our patients, we were able to collect multiple samples over time and noted some variation in thyrotropin level with time. Subsequent serum samples collected from three of these patients (patients 2, 3, and 4) were assessed in protocols A, B, and C. For patient 2, the measured thyrotropin values in the 3 assays were 12, 0.07, and less than 0.03 mU/L, respectively. Results for patients 3 and 4 showed essentially no change between protocols A and B. For patient 3, the values were 33, 33, and 8 mU/L, and for patient 4, the values were 8, 8, and 3.0 mU/L, respectively.

**DISCUSSION**

Artifactual immunoassay results caused by heterophilic antibody interference have the potential, if not suspected by the clinician or laboratory personnel, to cause inappropriate medical or surgical treatment or incorrect diagnosis that may lead to further unnecessary examinations. All immunoassays, especially two-site immunometric assays, may be prone to heterophilic antibody interference. The thyrotropin...
immunoassay is a good clinical model for examination of this form of interference because it is quantitatively the most important hormone assay in routine laboratory testing, particularly in light of recommendations that thyrotropin assay be used as a primary screen in the evaluation of thyroid function.9

We identified a negative bias of approximately 1 SD between protocols A and B. This bias was probably the result of matrix effects with the quantities of protein and immunoglobulin unavoidably being varied between the two methods.10 In our series, results for 41% of patients fell within ± 2 SD of the matrix effect–adjusted mean, and results for 70% of our patients fell within ± 3 SD. Previous studies have reported up to 40% of an unselected population having some demonstrable heterophilic antibody present,1 and our data, in which results for 30% of patients were more than ± 3 SD of the matrix effect–adjusted mean, confirm this assertion.

More important is the question of the incidence of heterophilic antibody activity in which the antibodies are present in such a concentration that they can cause clinically significant changes in the apparent result. With the observed matrix effect, we believe that it is difficult to be absolutely precise as to what constitutes a significant change. Reference to our difference plot in the Figure shows a small group of 6 patient samples in which the difference is more than 600%; in all cases the difference is greater than +13 SD. There is another group of 4 patients with samples with a difference of 150%, and with these patient samples, the difference is greater than +9 SD. We believe that at least these 10 samples reflect real and significant differences. This would reflect a prevalence of clinically significant heterophilic antibodies of 3.4% in this two-site ICMA that uses antibodies of ovine and murine origin. Generally, our results are comparable to the 9.1%,11 15%,1 and 3.1%12 observed for two-site assays using monoclonal antibodies. With respect to minimization of heterophilic antibody interference, these data suggest that in conventional two-site immunoassays, the use of antibodies from two different species may not confer a great advantage over those that use only monoclonal antibodies.

In our population sample, the added blocker in the kits tested overcame the presence of heterophilic antibodies and generated the “correct” answer. This is important information, indicating that the formulation of commercially available kits is appropriate.

A very small number of patient samples, collected over several months, contained such high heterophilic antibody concentrations that the blocking agents in the assay kits tested were overwhelmed. It is noteworthy that these patient samples were tested in several different thyrotropin assays and all of them showed qualitatively similar results. We found 7 patients in 21,000, a possible frequency of 0.03%. Some of these changes were very small and subtle, and it is likely that we have missed some. Thus, our estimate of the frequency of “rogue” samples with such high heterophilic antibody activity present that they will overcome blocking agents incorporated in kits, is a minimum estimate. To the best of our knowledge, no other data are available on the incidence of heterophilic antibody interference in currently available immunoassays, although recent reports confirm the presence of such patient samples.13,14

What should be done to identify and handle these rogue samples? They only can be identified on the grounds of apparently discordant results that require further investigation.15 Discordant results observed for thyroid function tests are those in which the usual inverse relationship between free thyroxine and thyrotropin is not evident, whereas for other analytes they are where the results are at variance with the clinical history. Our group of 7 patients (see the Table) had conditions previously described as associated with heterophilic antibody interference, ie, hyperthyroidism with a detectable thyrotropin level15–17 (patients 2, 5, and 6); unnecessary thyroxine therapy7,18,19 (patient 3); and OKT3 therapy20 (patient 7). Current clinical algorithms used for the diagnosis of thyroid hormone resistance syndromes and thyrotropin-secreting pituitary tumors include steps to exclude artifacts of thyroxine measurement but do not address problems of thyrotropin measurement.21,22 The results for patients 3 and 5 were not initially detected by the laboratory as unusual. The unusual result for patient 3 was detected when the thyroxine dosage was increased with a concomitant increase in free T4 and free T3, while the thyrotropin remained elevated (results not shown). Furthermore, the amount of sheep serum required to eliminate the heterophilic antibody interference increased with time for patient 3.16 The results for patient 5 are of concern because the results in the presence of heterophils were very believable. This latter result was only detected because the clinical staff insisted that the patient was in a thyrotoxic state. It is noteworthy that our assay can confidently distinguish these thyrotropin concentrations.4 Heterophilic antibody interference causing the observed thyrotropin level to be elevated above the reference range in patients in a thyrotoxic state is well documented (see previous discussion). We believe these cases (patients 2
and 5) are the first literature demonstration of heterophilic antibody interference resulting in such a small increase in measured thyrotropin as to be clinically misleading. These observations confirm the experimental predictions of the potential for incorrectly assigning biochemical euthyroid status to hyperthyroid patients based on blocked thyrotropin assays. It is interesting to note that in a recent study, the thyrotropin levels in one patient in a thyrotoxic state were less than 0.03 and 0.61 mU/L when measured by a monoclonal immunoradiometric thyrotropin assay and a monoclonal/polyclonal ICMA-thyrotropin, respectively. No explanation was offered for this discrepancy, but one possibility is a small level of heterophilic antibody interference in the ICMA-thyrotropin result. Our observations suggest that although the incidence of heterophilic antibody interference is small in modern blocked immunoassays, the potential for misdiagnosis exists for affected patients, particularly when thyrotropin is used as the primary screen for thyroid function.

We have shown that in approximately 3.4% of the population we studied, heterophilic antibodies were present in concentrations sufficient to potentially produce discordant patient results. However, the architecture of currently available kit assays, with added blocking agents, is sufficient to deal with nearly all patient samples with heterophilic antibody activity present. Only when the heterophilic antibody concentration is extremely high are potentially misleading patient results likely to be reported to the clinician. In these latter cases, only careful perusal of results before reporting them will enable identification of samples with potentially high heterophilic antibody concentrations. Once identified, addition of extra homologous serum or antisera can determine whether these samples are in fact rogue samples or true results.

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REFERENCES