Misleading results from immunoassays of serum free thyroxine in the presence of rheumatoid factor

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A novel interference with measurements of serum free thyroxine (FT4) caused by rheumatoid factor (RhF) is described. We found misleading, sometimes gross, increases of FT4 results in 5 clinically euthyroid elderly female patients with high RhF concentrations. All 5 patients had high FT4 on Abbott AxSYM® or IMx® analyzers. “NETRIA” immunoassays gave misleading results in 4 of the 5 patients; Amerlex-MAB® in 2 of 4 patients; AutoDELFIA® in 2 of the 5; and Corning ACS-180® and Bayer Diagnostics Immuno 1® in 1 of the 5. BM-ES700® system results for FT4 in these women remained within the reference range. Results for serum T4, thyroid-stimulating hormone, free triiodothyronine, thyroid-hormone-binding globulin, and FT4 measured by equilibrium dialysis were normal in all 5 patients. Drugs, albumin-binding variants, and anti-thyroid-hormone antibodies were excluded as interferences. Addition to normal serum of the RhF isolated from each of the 5 patients increased the apparent FT4 (Abbott AxSYM). Screening of 83 unselected patients demonstrated a highly significant positive correlation between FT4 (Abbott AxSYM) and RhF concentrations. Discrepant, apparently increased FT4 with a normal result for thyroid-stimulating hormone should lead to measurement of the patient’s RhF concentration.

INDEXING TERMS: hyperthyroidism • rheumatoid arthritis • fluoroimmunoassay • polyethylene glycol • affinity chromatography • immunoabsorption • equilibrium dialysis • thyroid-stimulating hormone

Diagnosis and management of patients with suspected thyroid disease depend on reliable measurements of serum concentrations of thyroid hormones and thyroid-stimulating hormone (TSH).6 These measurements have become readily available through the widespread use of automated immunoassays. Measurements of “free” thyroid hormones, particularly free thyroxine (FT4), are central to the TSH/FT4 testing strategy [1, 2]. Analytical interferences with measurements of serum FT4 assays are well recognized and include the presence of anti-thyroid hormone antibodies, thyroid-hormone-binding albumin variants (found in familial dysalbuminemic hyperthyroxinemia), heterophile antibodies, and various drugs (including salicylate, for some methods) [3–6]. The frequency with which the different assay methodologies in use are affected is not precisely known [2, 4].

Stimulated by the finding of a spuriously increased FT4 result in one elderly female patient, we surveyed prospectively the thyroid-screening workload of our general hospital laboratory for similar problems. Over a 6-month period we found another four patients with a similar pattern of results. All five patients were elderly women with high concentrations of serum rheumatoid factor (RhF). A comparison of our FT4 results in these patients with results from other laboratories using similar methods demonstrated widespread interference with FT4 measurements.
**Materials and Methods**

Serum FT₄ in the five patients described were measured with the Abbott Diagnostics systems, all within 4 h of blood collection; FT₄ in serum stored at 4 °C was stable for at least 48 h. Sera from these patients were also stored at −80 °C in 1-mL aliquots for the further studies described. The aliquots were thawed only once and kept for no longer than 24 h at ambient temperature before analysis for FT₄ in other analytical systems or for other thyroid-related hormones and proteins; RhF was isolated from parallel aliquots within ~2 h after thawing. The survey of patients’ sera with positive RhF was performed on specimens frozen at −20 °C for as long as 3 months before assay. Procedures followed for obtaining patients’ samples were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

TSH was measured by the following techniques: AxSYM® (Abbott Diagnostics Div., Berks., UK); TSH MAIAclone® IRMA (cat. no. 370023; Bio-Stat, Stockport, UK); “NETRIA” 2-step immunoenzymometric assay [North-East Thames Regional Immunoassay (NETRIA) Laboratory, London, UK]; AutoDELFIA® (Wallac UK, Milton Keynes, UK); ACS-180® (Ciba Corning Diagnostics, Halstead, Essex, UK).

RhF was isolated from patients’ sera as follows, all steps done at room temperature: We dialyzed 1-mL serum aliquots for 5 h against 2 L of 10 mmol/L sodium phosphate buffer, pH 7.4, containing KCl, 2.7 mmol/L, and NaCl, 500 mmol/L (“0.5 M PBS”). We then applied 0.5 mL of the dialyzed serum to 0.8 × 5 cm columns of human IgG immobilized on agarose (Sigma–Aldrich, Poole, UK; cat. no. A-6284, lot 07H8824; 5–10 mg of IgG bound per milliliter of settled gel). The eluent was “0.5 M PBS” containing bovine albumin, 10 g/L, and the flow rate was 0.1 mL/min. We applied the dialysate by washing it onto the column with 0.25 mL of eluent and stopping the flow for 15 min; the first 5 mL of eluate following the sample application was collected. We then applied to the column freshly prepared potassium thio-cyanate, 3 mol/L in “0.5 M PBS” containing 10 g/L bovine albumin (“3 M KSCN”), and collected the next 5 mL of eluate, which contained most of the RhF activity. We promptly dialyzed both 5-mL eluate fractions exhaustively against 10 mmol/L sodium phosphate buffer, pH 7.4, containing KCl, 2.7 mmol/L, and NaCl, 138 mmol/L (phosphate-buffered saline; PBS), and concentrated the dialysates to 0.5 mL in an Amicon (Stonehouse, UK) B15 concentrator. Inclusion of bovine albumin in the eluents was essential for good recovery of RhF. Serum protein electrophoresis and immunofixation was performed on the Paragon system [Beckman Instruments (UK), High Wycombe, UK] as recommended by the manufacturer.

To measure RhF in eluates from IgG-agarose columns, we used the Beckman Array 360®, having diluted 0.05 mL of each eluate 1:5 in PBS containing 10 g/L bovine albumin. To measure the apparent FT₄-increasing activity of the RhF in eluates from IgG-agarose columns, we mixed 0.125 mL of “normal” serum (serum without high concentrations of RhF or FT₄) with 0.125 mL of the concentrated eluates from IgG-agarose, eluted with either “0.5 M PBS” or “3 M KSCN,” from each of the five patients and from a “normal” serum and then measured the FT₄. Endogenous FT₄ concentrations in the eluates, measured by mixing 0.125 mL of each dialyzed concentrated eluate with an equal volume of PBS, were negligible. Apparent FT₄-increasing activity was calculated as: (apparent FT₄ in the mixture of patient’s eluate and “normal” serum) − (apparent FT₄ found in the corresponding eluate from the “normal” serum).

**Patients**

Patient 1 was an 84-year-old woman with rheumatoid arthritis that was controlled with enteric-coated prednisolone, 2.5–5 mg per day. Her case was reviewed in the outpatient department because of weight loss of ~5 kg in the previous 6 months. Marked rheumatoid deformities of both hands was evident but no signs of thyrotoxicosis [2]. This patient, like the other patients reported here, had never received medication with thyroid preparations. Thyroid-function tests in March 1995 showed an increased serum FT₄ inconsistent with the normal TSH concentration (Table 1). Review of her thyroid-function tests performed over the previous 3 years (1992–1995) showed markedly high FT₄ concentrations with normal values for TSH (Fig. 1). Concern about the inconsistent thyroid-function results led to reinvestigation of this patient (Tables 1 and 2). Results of liver-function tests, serum protein electrophoresis, immunoglobulins (G, A and M), thyroid-hormone-binding globulin, anti-thyroid-microsomol and anti-thyroglobulin antibodies, screen for familial dysalbuminemic hyperthyroxinemia, and anti-T₄ and anti-T₃ antibodies were normal.

Four additional patients with persistent isolated unexplained increases of FT₄ were found during a prospective 6-month survey (Table 1) of ~9100 combinations of FT₄/ TSH screening results, of which 1365 were from women older than 70 years. Four of the five patients received steroid medication, three of whom were taking prednisolone, <10 mg/day. All patients had normal concentrations of thyroid-binding globulin, a negative screen for familial dysalbuminemic hyperthyroxinemia, and no anti-T₄ and anti-T₃ antibodies.

**Results**

In addition to the assays stated above, serum from each of the five patients was also analyzed for FT₄ with the Abbott IMX® analyzer and was sent to colleagues in other laboratories for analysis of FT₄ by other analytical systems (Table 2). The results demonstrate marked analyzer-dependent variation. Depending on the analytical system in use by the laboratory, a patient’s FT₄ result might vary from normal (consistent with the measured TSH) to increased by >400%.

In contrast, measurements of TSH in each of the
patients shown in Table 1 and by the four quite different assay systems described in Materials and Methods did not show marked analyzer-dependent variation. All 5 patients had TSH concentrations within the assay reference range for all 5 different TSH methods examined, except for one measurement of TSH in Patient 3, which demonstrated a borderline increase (5.19 mU/L) compared with an upper reference limit of 5.0 (Table 1).

Because the five patients all had high concentrations of RhF, we assayed additional subjects’ samples to examine whether there was any general relation between the FT4 measured by immunoassay and the RhF concentrations in serum (Fig. 2). The criteria for inclusion of patients were a positive RhF by immunonephelometry (>20 kU/L), a normal TSH concentration, and clinical absence of thyroid disease. The results shown in Fig. 2 (which exclude the
five original patients) demonstrate a highly significant statistical association between apparent FT4 and concentrations of RhF (Spearman rank correlation coefficient \( r = 0.53, P < 0.001 \)).

To demonstrate directly that RhF was the cause of the misleading results we had found, we depleted of RhF the sera from each of the five patients originally studied, using immunoadsorption on columns of IgG-agarose. The RhF bound to each column was eluted with a chaotropic solvent, 3 mol/L potassium thiocyanate, and was recovered in the yields listed in Table 3. The RhF-depleted and the purified RhF fractions were tested for their ability to increase the apparent FT4 measured in normal serum (Fig. 3). Preliminary experiments had demonstrated that mixing normal serum with the sera from each of the 5 patients would lead to an apparent FT4 increase.

Most of the apparent FT4-increasing activity in each serum was associated with the IgG-agarose-bound fraction, which is consistent with RhF being the cause of the activity (Fig. 3). Electrophoresis of the fractions eluted from IgG-agarose from patient 5 showed that the monoclonal IgA component was not bound to the column (Fig. 3, top).

**Table 2. FT4 measurements (pmol/L) in the 5 patients by 6 different immunoassays and equilibrium dialysis.**

<table>
<thead>
<tr>
<th>Immunoassay system*</th>
<th>Principle</th>
<th>Reference rangeb</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbott AxSYM</td>
<td>2-step fluoroimmunoassay</td>
<td>9–24</td>
<td>44</td>
<td>40</td>
<td>39</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>Abbott IMx</td>
<td>2-step fluoroimmunoassay</td>
<td>9–24</td>
<td>31</td>
<td>31</td>
<td>37</td>
<td>29</td>
<td>40</td>
</tr>
<tr>
<td>NETRIA EIA</td>
<td>2-step enzyme immunoassay</td>
<td>10–22</td>
<td>36</td>
<td>34.2</td>
<td>38</td>
<td>21.7</td>
<td>78.1</td>
</tr>
<tr>
<td>NETRIA EIA + PEG</td>
<td>As above, after treating serum with 200 g/L PEGc</td>
<td>10–22</td>
<td>19.0</td>
<td>22.9</td>
<td>16.2</td>
<td>19.0</td>
<td>27.2</td>
</tr>
<tr>
<td>Amerlex MAB</td>
<td>Competitive labeled antibody, 1-step RIA</td>
<td>12–24</td>
<td>68</td>
<td>—</td>
<td>17.4</td>
<td>20.4</td>
<td>26.8</td>
</tr>
<tr>
<td>AutoDELFA</td>
<td>2-step fluoroimmunoassay</td>
<td>8.5–19</td>
<td>17.9</td>
<td>23.9</td>
<td>14.6</td>
<td>18.5</td>
<td>24.9</td>
</tr>
<tr>
<td>Corning ACS-180</td>
<td>Analog: 1-step immunoassay</td>
<td>9–24</td>
<td>17.7</td>
<td>21.1</td>
<td>15.6</td>
<td>16.3</td>
<td>106, 56.2d</td>
</tr>
<tr>
<td>Corning + PEG</td>
<td>As above, after treating serum with PEG</td>
<td>9–24</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>24</td>
</tr>
<tr>
<td>Bayer Immuno 1</td>
<td>2-step enzyme immunoassay</td>
<td>9–24</td>
<td>17.8</td>
<td>26.5</td>
<td>18.4</td>
<td>19.0</td>
<td>22.2</td>
</tr>
<tr>
<td>Boehringer Mannheim</td>
<td>ES700</td>
<td>2-step analog enzyme immunoassay</td>
<td>10–26</td>
<td>17.2</td>
<td>21.6</td>
<td>16.2</td>
<td>18.2</td>
</tr>
<tr>
<td>Equilibrium dialysis (FT4-ED); Nichols</td>
<td>Physical separation of free hormone, then immunoassay</td>
<td>10–36</td>
<td>29.9</td>
<td>29.8</td>
<td>21.4</td>
<td>29.8</td>
<td>24.8</td>
</tr>
</tbody>
</table>

* In addition to those listed in text, manufacturers were: Amerlex MAB®, Johnson & Johnson Clinical Diagnostics (Amersham, Bucks., UK); Immuno 1®, Bayer Diagnostics (Newbury, Berks., UK); ES700®, Boehringer Mannheim UK (Lewes, East Sussex, UK); FT4-ED, Nichols Institute Diagnostics (Newport, Essex, UK)

b Patients’ results outside reference ranges are in **bold** type.

c Equal volumes of serum and 400 g/L polyethylene glycol 6000 (PEG) are incubated at room temperature for 1 h; the specimen is then centrifuged at 1000g for 10 min and the supernatant is assayed.

d Measurements from two independent laboratories.

—, not measured.
Discussion

All five patients reported had a consistently high FT₄ value in at least two of the eight routine immunoassays examined; serum from one patient (patient 5) gave abnormal results in six of the assay systems. FT₄ results by equilibrium dialysis, the “gold-standard” method for FT₄, were normal in all patients (Table 2) and consistent with the measurements of TSH [6]. The presence of RhF at high concentrations in all five sera is almost certainly the cause of the interference. This is supported by the following observations: All five patients had high concentrations of RhF (Table 1); precipitation with polyethylene glycol, a procedure known to precipitate immunoglobulins, virtually abolished the interference (Table 2); and the apparent increase of FT₄ in normal serum was almost entirely confined to the IgG-agarose-bound fraction of each patient’s serum, i.e., the fraction containing purified RhF (Fig. 3).

Reported causes of interference with FT₄ assays include: thyroxine-binding albumin variants [7]; the presence of heterophile antibodies, e.g., human anti-mouse antibodies [3]; nonspecific immunoglobulins reacting with the assay system [6]; and anti-T₄ and anti-triiodothyronine (T₃) antibodies [1, 3, 5]. Immunoglobulin-mediated interferences in commercial and noncommercial assays are minimized by adding nonimmune globulin from the same or related species as the antibody used for the assay (“blockers”); nonetheless, such interferences in immunoassays still occur in individual patients [9].

The principle of the Abbott AxSYM and IMx assays for FT₄ (Abbott Reference Data List 7A54 (July 1994) and 2222 (Dec. 1990)) is the initial mixing of microparticles coated with sheep anti-T₄ antibody and the patient’s serum. A relatively low concentration of sheep antibody is used so that the equilibrium between free and bound hormone in the patient’s serum is perturbed as little as possible. After washing, alkaline phosphatase-labeled T₃ is added to the solid phase and occupies any vacant anti-T₄ binding sites. The fluorescence signal after further washing and adding of a fluorogenic substrate has an inverse relationship to the FT₄ concentration in the patient’s serum. We hypothesize that, in the Abbott AxSYM assay, RhF reacts with the immobilized sheep anti-T₄ and inhibits the subsequent binding of alkaline phosphatase-labeled T₃. The fluorescence signal will therefore be decreased and the apparent FT₄ concentration will be increased. Immobilization of the sheep immunoglobulin may make it reactive with the human RhF in a similar way as immobilization of human IgG on agarose, which we used to purify RhF from the patients’ sera (Fig. 3). Inhibition of the binding of reagents by anti-T₄ immunoglobulin may also underlie the interferences seen in the other system in Table 2. Interspecies binding of RhF is well recognized [10, 11].

Interestingly, in Patient 5, who had a low concentration of a serum paraprotein (Table 1), neither the RhF activity nor the apparent FT₄-increasing activity was associated with the paraprotein (Fig. 4 and Table 3). The presence of the paraprotein appears to be coincidental.

We are surprised that interference from RhF in these...
assays has not been previously reported, even in studies of thyroid function in rheumatoid arthritis [12]. Presumably, this is explained by the variable incidence of the problem among analyzer systems (Table 2) and the finding that only a minority of RhF-positive patients’ sera have an apparent FT4 above the upper reference limit (Fig. 2). Furthermore, the laboratories that use only ultrasensitive TSH assays as a front-line screen for thyroid status (a widely used strategy in the UK) will, of course, not detect such patients. All five patients reported here were elderly women, a group with a high incidence of autoantibodies; one, however, patient 3, did not have clinical rheumatoid arthritis despite a high RhF concentration. Furthermore, RhF was measured in two of these patients only after the misleading FT4 was discovered and then only as part of this investigation. Because RhF may occur even in healthy individuals as well as in patients having a wide variety of nonrheumatological diseases [10, 13], the recognition of RhF or associated immunoglobulins as a cause of misleading FT4 results may be difficult. The exact incidence of this novel interference among patients screened for thyroid disease remains to be determined.

In conclusion, our findings reinforce recent recommendations [4] for more rigorous assessment of interferences in commercial diagnostic systems for FT4, which are among the most widely used of all immunoassays. Indeed, we expect that RhF interferences with other immunoassays will be found. We recommend that patients found to have discrepant increases of serum FT4 concentrations and a normal TSH value should be screened for RhF. Clearly, however, even this strategy will fail to detect misleading FT4 results if the result is increased only into the reference range, rather than above the upper limit of the range.

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References