To the Editor:

Hemochromatosis is a common autosomal recessive genetic disorder of iron metabolism. In the United Kingdom, more than 90% of patients with hereditary hemochromatosis are homozygous for the C282Y mutation of the \textit{HFE} gene, but other single-nucleotide polymorphisms (SNPs) within the \textit{HFE} gene, namely H63D and S65C, have also been associated with the hemochromatosis phenotype. Various PCR-based methods can detect these SNPs, including a multiplex PCR for the two common SNPs, C282Y and H63D (1). This method involves PCR-mediated site-directed mutagenesis for C282Y and H63D to create a BbrPI restriction site in the wild-type PCR products. The presence of polymorphic alleles for both C282Y and H63D abolishes the restriction site so that the mutated allele remains undigested.

Using this method (1), we found two family members who appeared to be homozygous for H63D, but another laboratory had found these individuals to be heterozygous. DNA sequencing confirmed heterozygosity for H63D and, in addition, heterozygosity for S65C. We postulated that the presence of the S65C polymorphism interfered with the BbrPI restriction enzyme site and analysis of the H63D polymorphism by our method (1).

We reanalyzed 250 patients for H63D and S65C, using an alternative PCR method (2) with the same forward primer but a different reverse primer and producing a 174-bp product. The H63D genotype was determined by use of \textit{bcl-1} restriction enzyme digestion, which produced two fragments of 104 and 70 bp in the mutated allele and an undigested product in the wild-type allele. The S65C genotype was determined by use of the same PCR for H63D but with a \textit{Hinf-1} restriction enzyme digestion producing two products of 113 and 61 bp in the wild-type allele but an undigested product with the mutated allele.

According to the multiplex PCR (1), 4% were homozygous H63D and 19% were heterozygous; 4.8% were heterozygous S65C and none were homozygous. By the alternative PCR method (2), six cases that had been genotyped as homozygous for H63D by the multiplex PCR method (1) were found to be compound heterozygotes for H63D and S65C. This was confirmed by DNA sequencing.

The presence of the S65C mutated allele interferes with detection of the H63D genotype by the multiplex method of Stott et al. (1). This errorously leads to $\sim$1–2% of tested individuals being labeled as homozygous for H63D when they are in fact heterozygous for H63D. The presence of the S65C allele appears to lead to a failure to produce the expected digestion products. In all six such patients, an undigested PCR product was produced, indicating that it was not a failure of PCR but a failure of digestion. In four other patients who were heterozygous for S65C and did not have H63D complete digestion at the BbrPI site did occur. This failure of digestion occurred only in those S65C-heterozygous patients who were heterozygous for H63D.

The S65C SNP is outside of the restriction site and would not be expected to interfere with digestion by BbrPI according to available information on the restriction enzyme site. One possible explanation is that methylation occurs to prevent restriction enzyme digestion in this region. Such methylation, however, would have to be highly specific for this DNA sequence.

A recent UK NEQAS survey indicated that laboratories in the United Kingdom are using many different PCR methods for detecting the common hemochromatosis polymorphisms, and several use the multiplex PCR method (1). Unfortunately, this multiplex method may lead to error in up to 2% of samples. Our current practice is to test for all three mutations.

References


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Aberrant Thyroid Testing Results in a Clinically Euthyroid Patient Who Had Received a Tumor Vaccine

To the Editor:

We describe here the first case of immunoassay interference from
human anti-mouse antibodies (HAMAs) induced by a therapeutic anti-idiotypic antibody. A 76-year-old woman was referred to the Emory Endocrinology Clinic in May 2002 for evaluation of abnormal thyroid function tests. Between August 2001 and March 2002, her total triiodothyronine (T₃) was 6.62 to >12.32 nmol/L, but her T₄ uptake, total thyroxine (T₄), free T₄, thyroid-stimulating hormone (TSH), and thyroglobulin were all abnormally high, whereas TSH was low. Only anti-thyroglobulin antibody was within the reference interval. At a reference laboratory, all thyroid test results were normal except total T₃, measured by RIA, which was high. Anti-T₃ and anti-T₄ antibodies were not detected. At another local hospital, results for thyroid assays performed on the Abbott AxSYM were within the reference intervals except for TSH, which was below the reference interval.

Excess exogenous thyroid hormone ingestion and endogenous production giving rise to these thyroid testing aberrations were considered highly unlikely based on clinical history and evaluation of the patient. Exogenous T₃ overdose could account for the high total T₃ results detected by RIA and the Beckman Coulter ACCESS, but would fail to explain why total T₄ and free T₄ were also increased. Likewise, T₃ toxicosis was compatible with the assay results from the ACCESS Immunoassay System, but a euthyroid clinical picture was highly inconsistent with this possibility. 5-Fluorouracil administration can increase serum thyroid-binding globulin concentrations, which may in turn increase total T₃ and total T₄ values (2). However, this theory could not explain the abnormal concentrations of the free hormones.

The possibility of immunoassay interference was next considered. Autoantibodies against thyroglobulin, T₃, and T₄ have been documented in the setting of hypothyroidism, hyperthyroidism, and goitrous states and may be present in up to 1.8% of the euthyroid general population (3–5). Tests for these autoantibodies were negative in our patient, however; thus, these autoantibodies were ruled out as a possible cause of the aberrant results. Even when anti-T₃ or anti-T₄ antibodies are present, they may not necessarily cause analytical interference in immunoassays, as do other autoantibodies (5, 6). On the other hand, anti-animal antibodies are increasingly being recognized as a source of immunoassay interference. Some of these patients do not have well-documented histories of animal antigen exposure (7).

We retested our patient’s serum after treatment with heterophile blocking tubes (Scantibodies Inc.). When 0.5 mL of the patient’s serum was added to the blocking tube, complete corrections of free T₄ and thyroglobulin were achieved on the ACCESS. The other thyroid indices, with the exception of total T₄, were also partially corrected, although still above the upper limits of their corresponding reference intervals. When 0.25 mL of serum was used (doubling the ratio of blocking reagent to serum), all thyroid indices (except total T₄) fell within the reference intervals. Total T₄ values, however, remained markedly increased at >377 nmol/L (>29.2 μg/dL).

The investigational tumor vaccine (CeaVac) that our patient received is a monoclonal antibody with antigen binding sites that mimic CEA, thereby inducing an immune response against colon cancer cells producing CEA. As a monoclonal antibody derived from mice, CeaVac therefore has the propensity to induce HAMA formation.

HAMA interference is a potential pitfall for all immunoassays that use animal antibodies (7). In this case, the ACCESS total T₄ values failed to correct with the Scantibodies HAMA-blocking reagent. One possible explanation is that the specific HAMA in our patient’s serum was not reactive with the blocking reagent. Alternatively, the HAMA may

<table>
<thead>
<tr>
<th>Table 1. Thyroid testing results.</th>
<th>Reference laboratory</th>
<th>ACCESS</th>
<th>AxSYM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH, mIU/L (μIU/mL)</td>
<td>2.43 (2.43)</td>
<td>0.05 (0.05)</td>
<td>0.20 (0.20)</td>
</tr>
<tr>
<td>Total T₄, nmol/L (μg/dL)</td>
<td>148 (11.50)</td>
<td>&gt;377 (&gt;29.2)</td>
<td>137 (10.62)</td>
</tr>
<tr>
<td>Free T₄, pmol/L (ng/dL)</td>
<td>16.8 (1.30)</td>
<td>64.5 (5.00)</td>
<td>17.9 (1.39)</td>
</tr>
<tr>
<td>Total T₃, nmol/L (ng/dL)</td>
<td>11.6 (775)</td>
<td>8.2 (530)</td>
<td>1.7 (110)</td>
</tr>
<tr>
<td>Free T₃, pmol/L (pg/mL)</td>
<td>&gt;45.3 (&gt;29.4)</td>
<td>0.79 (79)</td>
<td></td>
</tr>
<tr>
<td>T₄ uptake, % (%)</td>
<td>0.79 (79)</td>
<td>0.79 (79)</td>
<td></td>
</tr>
<tr>
<td>Thyroglobulin, μg/L (ng/mL)</td>
<td>154 (154)</td>
<td>154 (154)</td>
<td></td>
</tr>
<tr>
<td>Anti-thyroglobulin, IU/mL</td>
<td>&lt;2.2</td>
<td>&lt;2.2</td>
<td></td>
</tr>
<tr>
<td>Anti-T₃</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-T₄</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values shown in parentheses are in conventional units.

* At the reference laboratory, total T₄ was measured by RIA (which gave a result above the reference interval), whereas the rest of the panel were measured on a Bayer ADVIA Centaur.

* Results were below the reference interval.

* Results were above the reference interval.
Increased Serum Concentrations of Intestinal Alkaline Phosphatase in Peritoneal Dialysis

To the Editor:

Patients suffering from chronic renal dysfunction are monitored by measurement of biochemical markers such as serum total alkaline phosphatase (ALP) to detect increased bone remodeling before the development of advanced renal osteodystrophy. There are at least five different isozymes/iso-enzymes of ALP: hepatic, skeletal, intestinal, placental, and tumor-associated (1). These can be separated by electrophoresis and differ in their resistance to heat inactivation, with placental and tumor-associated ALP being the most resistant. Approximately 95% of the total ALP activity in serum is derived from bone and liver sources; these isozymes occur in an ~1:1 ratio in healthy adults (2). Bone remodeling leads to release of skeletal ALP from osteoblasts and, hence, to increased total ALP activity in serum (1). However, one report has indicated that peritoneal dialysis per se may stimulate the intestine to secrete more intestinal ALP and intestinal variant ALP, leading to increased ALP activity in serum (3). I wish to report on two patients who were on long-term peritoneal dialysis and had moderately increased serum total ALP activity (2.4 and 4.4 μkat/L, respectively; cutoff value <1.8 μkat/L) without any biochemical signs of liver disease. Serum agarose gel electrophoresis followed by incubation of the gel with a chromogenic ALP substrate revealed a dominant band with electrophoretic mobility identical to that of intestinal ALP (Fig. 1). The band was unaltered by ficin-induced immunoglobulin fragmentation and disappeared after heat inactivation, excluding that it might represent immunoglobulin-bound macro-ALP or placental or tumor-associated ALP.

The two cases support the possibility of an association between peritoneal dialysis and increased serum concentrations of intestinal ALP and caution against interpreting increased total ALP activity in peritoneal dialysis patients as a sign of increased bone remodeling before further investigation by electrophoresis and/or determination of "bone-specific" ALP, for which immunochromatographic methods are available. It should in this context be noted that macro-ALP interferes with immunochromatographic tests for skeletal ALP (4). Thus, the presence of macro-ALP should be excluded by electrophoresis or an equivalent method in patients with increased serum concentrations of skeletal ALP.

The molecular mechanism behind the increase in intestinal ALP in peritoneal dialysis is unknown. There

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Fig. 1. Agarose gel electrophoresis of ALP isozymes/iso-enzymes in serum samples.

Lane 1, reference sample for hepatic ALP; lane 2, reference sample for skeletal ALP; lanes 3 and 6, untreated samples from patients 1 and 2, respectively; lanes 4 and 7, ficin-treated samples from patients 1 and 2, respectively (5 mg of ficin added to 100 μL of serum, followed by incubation at 37 °C for 30 min); lanes 5 and 8, heat-inactivated (65 °C for 5 min) samples from patients 1 and 2, respectively. Note the lipophilic appearance of the intestinal ALP band in lane 3, indicating that it might represent a previously described intestinal variant ALP with its membrane-binding domain retained (6). As expected, this domain is removed by treatment with ficin (lane 4).