Implications of Using Different Tissue Ferritins as Antigens for Ferritin in Serum: Four Radioimmunoassay Kits Compared

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We compared three serum ferritin radioimmunoassay kits and one noncommercial RIA for ferritin quantitation, with regression analysis of results for sera from 35 ostensibly healthy subjects. There was a good correlation (p < 0.001) between these various RIAs, but the slope of the regression line varied widely, most probably because of lack of standardization of the serum ferritin assay. Determination of the ferritin content of purified samples of tissue ferritin revealed that the kits differ in specificity, differences for purified human spleen ferritin and human liver ferritin being larger than those for normal sera. Removing the iron from purified liver ferritin increased antiseraum binding in two of the kits by twofold, but had no effect in two other kits. We conclude that commercial RIA methods for serum ferritin differ in specificity, and that this difference is related to the source of ferritin used in the production of the antibodies.

Additional Keyphrases: assessing iron status - errors resulting from lack of a generally accepted antigen standard - variation, source of - immunochemistry - "kit" methods

Estimation of ferritin in serum by radioimmunoassay (RIA) currently is widely used as a diagnostic tool. Low ferritin values are associated with iron deficiency. High ferritin concentrations (hyperferritinemia) can result from such conditions as iron overload, malignant disease, and tissue damage such as occurs in hepatitis (1, 2).

Ordinarily, ferritin in serum is heterogeneous, as has been demonstrated by isoelectric focusing of serum samples (3–5). In pathological conditions, however, a particular form of ferritin may predominate: decreased binding to concanavalin A (6), appreciable iron content (7–9), and anomalous reaction with antibodies to specific ferritins (10–12) have all been reported. Variations in the characteristics of ferritin in pathological sera may be related to its origin from a population of pathological cells. These tissue ferritins differ in iron content, isoelectric point, immunological properties, and subunit composition (13, 14).

Because commercial RIAs involve use of antibodies to ferritins from different tissues, purified by different methods, the antibodies supplied in the kits may have different specificities. We checked on this by comparing results obtained with three commercial RIA kits and one noncommercial kit for ferritin in sera from healthy subjects and for ferritins extracted from different tissues.

Materials and Methods

Materials

Phenylmethylsulfonylfluoride, benzamidine, and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO 63178; cadmium sulfate and thioglycolic acid from Merck, Darmstadt, F.R.G.; polyacrylamide gradient gels, molecular-mass markers for electrophoresis and Sepharose 6B from Pharmacia, Uppsala, Sweden; and reagents for dodecyl sodium sulfate electrophoresis from Bio-Rad Labs., Richmond, CA 94804. These and all other chemicals were of the highest quality available.

Serum Ferritin RIA kits

Commercial serum ferritin RIA kits were obtained from Clinical Assays, Division of Travenol Laboratories, Inc., Cambridge, MA 02139; Ramco Laboratories Inc., Houston, TX 77098; and Hoechst Pharma, Frankfurt, F.R.G. The noncommercial radioimmunoassay for serum ferritin ("Utrecht-kit") was developed at the University Hospital in Utrecht, The Netherlands, as described elsewhere (15). Table 1 lists the main features of these kits.

All procedures were done according to instructions supplied with the kits. In brief, the procedures were as follows:

Clinical Assays Kit: Mix a 100-μL serum sample with 100 μL of antiferritin antiserum and 500 μL of tracer and incubate for 3 h in a thermostated waterbath at 37 °C. Then add 500 μL of precipitating goat anti-rabbit IgG serum and continue the incubation for 30 min. Centrifuge all tubes in run at 1000 × g for 15 min, carefully decant the supernatant fluid, and measure the radioactivity of the pellet.

Ramco Kit: Place 10 μL of serum sample and 200 μL of in-

Table 1. Characteristics of the RIA kits for Serum Ferritin

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Antigen source</th>
<th>Reference interval for ferritin, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Assays</td>
<td>Competitive Liver</td>
<td>10–300</td>
</tr>
<tr>
<td>Utrecht</td>
<td>Sandwich Liver</td>
<td>8–200</td>
</tr>
<tr>
<td>Ramco</td>
<td>Sandwich Spleen</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Hoechst</td>
<td>Sandwich Liver/ placenta</td>
<td>30–185 (9)</td>
</tr>
</tbody>
</table>

* Competitive RIA methods are based on the competition between radiolabeled antigen (tracer) and unlabeled antigen (sample) for a limiting amount of antiserum; bound and unbound tracer are separated by addition of anti-rabbit IgG serum raised in goat ("second-antibody" method). In "sandwich" type RIA methods, antibodies immobilized on polystyrene tubes (Utrecht kit) or on microbeads (Ramco kit and Hoechst kit) are used to extract ferritin from the test serum, and the amount of extracted antigen is quantitated by the binding of radiolabeled antiferritin IgG. See text for sources. In the Hoechst kit, sheep antiserum to human liver ferritin was used for the antigen extraction step, and radiolabeled rabbit antiserum to human placental ferritin was used to quantitate the extracted antigen.

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cubation buffer in a tube containing one plastic bead coated with antiferritin IgG. After incubating for 6 h at room temperature, wash the bead two times and incubate subsequently with 200 μL of radiolabeled antiferritin IgG for 16 h at 4 °C. Wash the bead three times, then count its radioactivity.

Hoechst kit. To one plastic bead, coated with antiferritin IgG and suspended in 200 μL of buffer, add 100 μL of serum sample and incubate for 5 h in a thermostated waterbath at 37 °C. After washing the bead once, add 300 μL of radiolabeled antiferritin IgG and incubate the mixture overnight at 37 °C. Then wash the bead once more and count its radioactivity.

Utrecht kit. Place 100 μL of serum sample in tubes coated with antiferritin IgG, incubate for 18 h at 4 °C, then decant and wash the walls of the tubes four times with saline. Add 100 μL of radiolabeled antiferritin IgG to the tubes, and incubate for 4 h at room temperature. Decant, wash the tubes three times, then count their radioactivity.

Serum Specimens

The "normal" sera were prepared from blood collected from laboratory staff and from regular blood donors selected without conscious bias. The sera were divided into 1-mL samples, stored at −20 °C, and assayed within six months.

Procedures

Preparation of ferritins from various tissues. Tissue from human heart, liver, and spleen was obtained within 24 h postmortem, from the Department of Pathology, University Hospital, Utrecht. To prepare tissue ferritin extracts, we suspended 50 g of tissue in 50 mL of water to which benzamidine (1 mmol/L) and phenylmethylsulfonylfluoride (0.5 mmol/L) had just been added, then homogenized the suspension in a "Braun" blender. The particulate fraction was removed by centrifugation at 20 000 × g for 30 min.

To purify ferritin from the tissue homogenates, we used the following general procedure: heat treatment, 10 min at 73 °C; precipitation with half-saturated ammonium sulfate; ultra-centrifugation (100 000 × g, 180 min); ion-exchange chromatography on DEAE-Sepharose 6B and gel filtration on Sepharose 6B. The detailed procedure will be reported elsewhere (manuscript in preparation). We examined the purity of these ferritins by polyacrylamide electrophoresis, staining both for protein with Coomassie Brilliant Blue R-250, 1 g/L in ethanol/acetic acid/water (25/8/67 by vol), and for iron with potassium ferrocyanide, 10 g/L in methanol/acetic acid/water (30/7/63 by vol). The electrophoresis was carried out in 50 g/L polyacrylamide gels in lithium borate buffer (50 mmol/L, pH 8.6) or in polyacrylamide gradient (40–300 g/L) slab gels in a buffer containing, per liter, 90 mmol of Tris, 80 mmol of boric acid, and 903 mg of Na2EDTA, pH 8.4. All purified ferritin preparations contained a major band of $M_r = 450 000$ that stained both for protein and for iron. In the preparation from liver two minor bands of high molecular mass, representing ferritin oligomers, also stained for iron and protein.

High-$M_r$ liver ferritin. Protein eluting in the void volume of the gel filtration column was mainly ferritin oligomers. The electrophoretic pattern showed predominantly protein bands, which stained also for iron, having a mobility less than 10% that of the ferritin monomer band.

Iron withdrawal from liver ferritin. We dialyzed 1 mg of purified liver ferritin (in 1 mL) against 1 L of sodium acetate buffer (0.1 mol/L, pH 4.2) containing 0.1 mol of thioglycolic acid per liter. During the dialysis, the clear red-brown color of the ferritin preparation disappeared completely. After 24 h we replaced the dialysis fluid with 1 L of a mixture of diethyl barbiturate, 40 mmol/L, and NaCl, 0.1 mol/L (pH 6.8). This dialysis fluid was changed three times (total dialysis interval, four days). A control ferritin sample was prepared according to essentially the same procedure, but without thioglycolic acid. The ferritin preparations were subjected to polyacrylamide gradient slab gel (40–300 g/L) electrophoresis. The protein staining indicated a major band of $M_r = 450 000$ and one minor band, of high molecular mass, for both preparations. The bands stained also for iron in the control ferritin sample, while no iron staining was visible for apoferritin.

Natural liver apoferritin. We isolated apoferritin from partly purified liver ferritin that had not been subjected to ultracentrifugation. The ferritin was crystallized from a 200 g/L solution of CdSO4 and subjected to sucrose gradient centrifugation as described by Arosio et al. (8). The iron/protein ratio (by weight) of the apoferritin preparation was <0.005. The protein migrated as a single band ($M_r = 450 000$) in polyacrylamide gradient slab gels (40–300 g/L) with no visible staining for iron. After polyacrylamide slab gel (140 g/L) electrophoresis in dodecyl sodium sulfate under reducing conditions (18), we observed a single band, with apparent $M_r = 22 000$.

Preparation of ferritin samples for ferritin quantification by RIA. The samples were prediluted with a pH 8.0 solution containing, per liter, 50 mmol of diethyl barbiturate, 677 mmol of NaCl, 3 mmol of NaN3, 1 g of bovine serum albumin, and 1 mL of normal rabbit serum (17). We used the Utrecht kit to prescreen the ferritin concentration in the tissue preparations. For the last 10-fold dilution before assay we used the buffer supplied with the kit or recommended by the manufacturers. All samples were assayed in duplicate in two or three different dilutions. To compare results of the different kits, we assigned the sample dilution that gave a response that fell in the center of the standard curve, to circumvent possible problems of variable detection limits or deviations from linearity in the upper region of the assay limits.

Statistical Analysis

We compared results with the kits for control sera by using regression analysis according to the method of Deming and Morgan (18), which takes into account the error in both variables (19).

To examine whether a particular kit showed an abnormal sensitivity for a particular tissue ferritin, we used the following procedure: From the result of a tissue ferritin quantitation in "kit A" ($x$) we calculated which result was to be expected in "kit B" ($y$)predicted, based on the regression of the comparison between "kit A" and "kit B" for the control sera. The underlying assumption was that if two kits in a particular kit combination had comparable affinities, the values for serum ferritin and tissue ferritin should be indistinguishable. To test this hypothesis, we estimated the significance of the difference between the predicted $y$-value and the actually observed $y$-value by using the following formula (20):

$$t_{n-2} = \frac{|y - y_{\text{predicted}}|}{S_{y,x} \sqrt{\frac{1}{n} + \frac{\sum (x - \bar{x})^2}{S_x^2}}}$$

$S_{y,x}, S_x^2, \bar{x},$ and $n$ are derived from the regression of the control sera (see Table 2 for explanation of the symbols).

Other methods. Ferritin protein was measured according to Schalterle and Pollack (21). Ferritin iron was measured with bathophenanthroline according to Harris (22). Radioactivity was measured with a gamma counter (Trigamma 600; Baird Atomic Inc., Bedford, MA 01730).

Results

Quantitation of ferritin antigen in normal sera. The ferritin concentration in identical dilutions of a single normal
serum differed, depending on the kit used (Figure 1). Values were highest with the Hoechst kit, lowest with the Utrecht kit. These differences were maintained over the entire dilution range. Results with the kits were compared for 35–40 normal sera by regression analysis of results from the different kit combinations (Table 2). The correlation coefficients for the different kit combinations were highly significant (p < 0.001). The slopes of regression of the kit combinations substantiated the quantitative differences between the kits (Figure 1). The Hoechst kit showed the largest variability, as judged from the large deviation of regression in all combinations with the Hoechst kit (Table 2).

Quantitation of ferritin antigen in kit standards and in tissue preparations. We examined whether the antisera used in the kits had different specificities. Ferritin antigen was quantified in kit standards, tissue extracts, and purified tissue ferritins (Table 3). The kit standards responded almost identically as for the normal sera except that the Utrecht kit was less sensitive for the Ramco standard. Also, ferritin measured in tissue extracts did not reveal large differences among the kits when compared with normal sera. Purified ferritins, however, showed considerable variation when assayed with the different kits. A significantly higher sensitivity for the Ramco and Hoechst kit was observed against purified liver and spleen ferritin preparations. Moreover, the Hoechst kit showed an unusually high reactivity for the more aggregated forms of purified liver ferritin. In contrast, purified heart ferritin did respond like serum ferritin in all kit combinations. Liver apoferritin also responded more like serum ferritin, except for a higher sensitivity of the Utrecht kit for native apoferritin.

Effect of removal of iron on ferritin antigen quantitation. Purified liver ferritin was depleted of iron by dialysis against thioglycolic acid. The immunoreactivity of the iron-poor ferritin was increased in the Utrecht and Clinical Assays kits but not in the Ramco and Hoechst kits (Figure 2).

Discussion

There was a good correlation among the three commercial radioimmunoassay kits and one noncommercial kit for ferritin determination in normal serum. Absolute values, however, varied widely, reflecting the lack of standardization of serum ferritin determinations. We have demonstrated here that the differences between the kits cannot be explained solely on the basis of quantitative differences. Purified ferritins, especially from liver and spleen, demonstrated much larger differences between the kits than could be explained with the differences found in normal sera. Moreover, the effect of removal of iron

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**Table 2. Comparison of Serum Ferritin RIA Kits Values for Control Sera**

<table>
<thead>
<tr>
<th>Kits compared b</th>
<th>( b_0 )</th>
<th>( b_1 )</th>
<th>( S_{xy} )</th>
<th>( \bar{x} )</th>
<th>( S_x^2 )</th>
<th>( r^2 )</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utrecht–Clinical Assays</td>
<td>2.7</td>
<td>1.56</td>
<td>2.77</td>
<td>9.3</td>
<td>24.2</td>
<td>0.91</td>
<td>35</td>
</tr>
<tr>
<td>Utrecht–Ramco</td>
<td>1.9</td>
<td>1.01</td>
<td>2.09</td>
<td>9.3</td>
<td>24.2</td>
<td>0.86</td>
<td>35</td>
</tr>
<tr>
<td>Utrecht–Hoechst</td>
<td>10.7</td>
<td>3.01</td>
<td>8.31</td>
<td>9.2</td>
<td>24.6</td>
<td>0.83</td>
<td>34</td>
</tr>
<tr>
<td>Clinical Assays–Ramco</td>
<td>0.1</td>
<td>0.70</td>
<td>1.56</td>
<td>20.2</td>
<td>131.3</td>
<td>0.95</td>
<td>40</td>
</tr>
<tr>
<td>Clinical Assays–Hoechst</td>
<td>9.8</td>
<td>1.62</td>
<td>7.37</td>
<td>20.2</td>
<td>135.0</td>
<td>0.90</td>
<td>39</td>
</tr>
<tr>
<td>Ramco–Hoechst</td>
<td>8.5</td>
<td>2.38</td>
<td>5.59</td>
<td>14.4</td>
<td>63.8</td>
<td>0.95</td>
<td>39</td>
</tr>
</tbody>
</table>

a Sera from healthy laboratory staff and from regular blood donors were assayed for serum ferritin according to the instructions of the manufacturers. All the serum ferritin values fell within the specified normal ranges of the kits. The assay of the serum samples was allocated over three different assay runs. Each sample was assayed in duplicate in one run for all four kits in the same week. The symbols represent: \( b_0 \): intercept of the linear relationship between \( x \) and \( y \); \( b_1 \): slope of the linear relationship between \( x \) and \( y \); \( S_{xy} \): deviation from regression; \( \bar{x} \): mean of the \( x \) data set; \( S_x^2 \): variance of the \( x \) data set; \( r^2 \): correlation coefficient; \( n \): number of sera tested. b First kit named as \( x \), second kit as \( y \).

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**Table 3. Quantitation of Ferritin Antigen in Kit Standards and Tissue Ferritin Preparations**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Utrecht k</th>
<th>Clinical Assays k</th>
<th>Ramco k</th>
<th>Hoechst k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utrecht standard</td>
<td>15</td>
<td>27</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Clinical Assays standard</td>
<td>15</td>
<td>20</td>
<td>17</td>
<td>46</td>
</tr>
<tr>
<td>Ramco standard</td>
<td>16</td>
<td>16</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>Hoechst standard</td>
<td>15</td>
<td>32</td>
<td>18</td>
<td>68</td>
</tr>
<tr>
<td>Liver extract</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Spleen extract</td>
<td>10</td>
<td>11</td>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>Heart extract</td>
<td>16</td>
<td>19</td>
<td>10</td>
<td>49</td>
</tr>
<tr>
<td>Liver ferritin</td>
<td>5</td>
<td>8</td>
<td>16</td>
<td>73</td>
</tr>
<tr>
<td>(oligomers)</td>
<td>22</td>
<td>30</td>
<td>68</td>
<td>445</td>
</tr>
</tbody>
</table>

Ferritin standard solutions and tissue ferritins were prepared, diluted, and assayed as described in Materials and Methods. The assay of the samples was allocated over three different assay runs. Each sample was assayed in one run for all four kits in the same week. Means of duplicate measurements are shown. The mean coefficients of variation, as estimated from 50 samples measured in duplicate, were: Utrecht 7.4%; Clinical Assays 8.8%; Ramco 5.8%, and Hoechst 10.1%. The results obtained with the various kits were compared for all possible kit combinations (see Table 2). Those samples for which a particular kit, as indicated by the column headings, showed an abnormal sensitivity (p < 0.001) are marked by superscripts. The superscripts, in turn, designate the kit against which the particular kit was compared ("Kit A" and "Kit B," respectively; see Statistical Analysis section in Materials and Methods). C. Clinical Assays, R. Ramco, H. Hoechst.
Fig. 2. Effect of removal of iron on the immunological reactivity of ferritin

The ferritin content in a purified liver ferritin sample (white bar) and in an iron-poor ferritin sample (apo-ferritin, cross-hatched bar) derived from the same source was quantified in duplicate in one run for all four kits. This ferritin preparation was purified from a different liver from those listed in Table 2

had no effect on ferritin antigenicity in two of the kits while the two other kits showed a twofold increase in antisemum binding.

Our results for normal sera are consistent with previous reports where other kits for the measurement of serum ferritin were compared (23-27). These investigators also found considerable quantitative differences, which were ascribed to the lack of ferritin standardization. However, this will be of limited help for ferritin quantification in pathological sera. Here the ferritin composition may be altered depending on the disease and the tissue origin of ferritin. Indeed, we found some pathological sera that responded more like liver ferritin in the kit comparison (F. L. A. Willekens and B. A. van Oost, manuscript in preparation).

As for the response of tissue ferritins, it should be stressed that we did not attempt to quantify the response of tissue ferritins in terms of antisemum binding per milligram of protein of the tissue ferritins. Protein determination of tissue ferritins can be erroneous (28). To circumvent this problem we chose to compare the response of the same amount of ferritin protein in the different RIA kits. The divergent response of tissue ferritin preparations is not consistent with the concept that differences in tissue ferritin antigenicity are related to their isoelectric point (13). Although natural apoferritin and heart ferritin reportedly (11) consist of basic isoferritins and mainly acidic isoferritins, respectively, both ferritin preparations responded much like serum ferritin when measured by the different kits.

The different procedures used to isolate tissue ferritins may better explain the observed discrepancies among tissue-ferritin preparations. Ferritin purified by CdSO₄ crystallization such as the human liver ferritin standard of the Clinical Assays kit, the human spleen ferritin standard of the Ramco kit, and the natural apoferritin preparation all responded like serum ferritin in the kit combinations. This is in harmony with another study, in which crystalline liver and spleen ferritin were shown to respond identically in a sandwich-type RIA for serum ferritin (29). The unusually high sensitivity of the Hoechst kit for liver ferritin may be in part due to trapping of ferritin oligomers in the antisemum-coated beads in this assay, causing excessive binding of radiolabeled antisemum. In the Ramco kit, however, in which antisemum-coated beads are also used, the effect was less dramatic.

Porter (30) demonstrated an inverse relationship between ferritin iron content and ferritin reactivity in a competitive RIA in which crystalline human spleen ferritin was the standard. We found the same trend with the Clinical Assays kit and the Utrecht kit but not with the Hoechst and Ramco kits. This is another indication that the kits differ in their reactivity for the various antigenic sites on ferritin.

In conclusion, the observed differences between RIA kits for ferritin in normal serum could be explained by quantitative differences in the kit standards. But in pathological sera with high ferritin content—in which a large contribution of tissue ferritin to the circulating ferritin may be expected—the different specificities of the RIA kits should also be considered.

We thank the manufacturers of the commercial serum ferritin RIA kits for making their products available without charge, and Dr. David Purdon for linguistic advice.

References


