

Immunologic Differences in Human Isoferritins: Implications for Immunologic Quantitation of Serum Ferritin

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Immunologic differences in several tissue isoferritin populations have been investigated using antibodies developed in rabbits against human liver ferritin and in guinea pigs against HeLa cell ferritin. Our results indicate that the quantity of ferritin measured by radioimmunoassays is mark-

edly affected by the isoferritin and subunit composition of the ferritin sample as well as by the nature of the antiferritin antibodies. Possible applications for the selective quantitation of different serum ferritin populations are discussed.

IT HAS BEEN RECOGNIZED for some time that small amounts of ferritin occur in serum in both normal and diseased states.¹⁻⁵ Interest in the quantitation of this ferritin has increased recently because of its potential as an index of iron stores⁶⁻⁸ and as a possible tumor marker.⁹⁻¹⁵ Although little is known of its source or whether it derives from cell damage or from secretion, serum ferritin apparently varies qualitatively as well as quantitatively in different physiologic states.^{5,14,16,17}

Qualitative differences may have important consequences for quantitation and hence for the reliability and diagnostic applicability of the assay. Most assays for serum ferritin involve immunologic procedures in which it is assumed that all ferritin molecules behave similarly. However, this thesis is not a valid assumption.^{10,17-20} These differences in immunologic behavior^{17,18} can be explained by observations that most tissue ferritins are not homogeneous populations, but families of structurally related molecules, or isoferritins.^{18,21-23} These isoferritins have a characteristic tissue distribution^{18,22} and appear to be hybrid molecules arising from various combinations of two, or possibly three, dissimilar subunit types of similar size.^{18,24} Variations in subunit composition account for immunologic differences among tissue ferritins.^{17,18}

This paper shows how the apparent level of various human ferritins in a commonly used radioimmunoassay (RIA) depends on their isoferritin and subunit composition and on the nature of the immunizing ferritin used for antibody production. We describe RIA systems which differentiate both qualitatively and quantitatively among different isoferritin populations. These assays may provide useful information about the type as well as amount of ferritin in biologic fluids in different pathologic states.

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MATERIALS AND METHODS

Ferritins. Human ferritins obtained from normal liver, heart, and HeLa cells were purified as described previously.^{21,22} Liver ferritin was crystallized with cadmium sulfate.²⁵ Natural apoferritin from liver was separated by ultracentrifugation in sucrose density gradients.¹⁷ Isoferritin profiles and isoelectric points of isoferritins were obtained by gel electrofocusing as described previously.¹⁷ Subunit compositions of ferritins were estimated after separation by acid-urea gel electrophoresis.^{18,24} Ferritin protein was estimated by the method of Lowry et al.²⁶ using bovine serum albumin (BSA) as a standard.

Antisera. Antibodies to crystalline human liver ferritin were raised in New Zealand white rabbits and to HeLa ferritin in Hartley guinea pigs as previously.^{17,18} The guinea pig antiserum was absorbed with increasing amounts of natural liver apoferritin until the antiserum no longer gave a reaction with it on Ouchterlony double diffusion or on counter-immunoelectrophoresis¹⁷ at levels up to 1 mg apoferritin/ml.

Goat anti-rabbit and rabbit anti-guinea pig immunoglobulins (Ig) were purchased from Calbiochem, Los Angeles, Calif., and from Miles Laboratories, Kankakee, Ill.

Iodination of Ferritin

Carrier-free ¹²⁵I was purchased from New England Nuclear, Boston, Mass. Chloramine T was obtained from Baker Chemicals, Philipsburg, N.J.

Liver ferritin was iodinated by incubating approximately 10 μg ferritin in 50 μl phosphate buffer (0.5 M, pH 7.5) with 1 mCi ¹²⁵I (< 5 μl) and 20 μg chloramine T at 22°C for 1 min. HeLa ferritin was iodinated in a similar manner except that it was incubated with only 10 μg chloramine T at 0°C for 60 min. Sodium metabisulphite (100 μg) was added to stop the reaction. Unbound ¹²⁵I was removed by passing the reaction mixture through Sephadex G-25 in a buffer containing 0.05 M Tris-HCl, pH 7.4, and 0.25% gelatin. Fractions containing ferritin were immediately diluted into 0.05 M Tris-HCl, pH 7.4, containing 1% BSA to give a working solution containing 250,000 cpm/ml and stored at 4°C.

Aliquots of all iodinated ferritins were incubated with serial dilutions of antibody, which were then precipitated with carrier immunoglobulins by a precipitating antiserum. At least 90%–95% of the total cpm in all iodinated ferritins used in the RIA procedures were immunoprecipitable. Preparations with lower immunoprecipitability were not used.

Radioimmunoassay Procedure

The procedure used was essentially that described by Marcus and Zinberg¹⁰ and by Niitsu et al.¹³ Known amounts of ferritin in a volume of 50 μl were incubated with the iodinated ferritin (50 μl), a limiting amount of antiferritin antiserum (100 μl) and 600 μl RIA buffer (0.02 M Tris-HCl, pH 7.4, 0.9% NaCl and 0.4% BSA) for 24 hr. The assay with liver antibodies used 1 ng of labeled liver ferritin (approximately 19,000 cpm) and 100 μl of the appropriately diluted antiserum (1:200,000, sufficient to precipitate 9000 cpm in the absence of competing ferritins). Because of the lower specific activity of the labeled HeLa ferritin, the assay with HeLa antibodies used about 10 ng labeled ferritin (approximately 9000 cpm) with 100 μl of absorbed antiserum (1:1000, which precipitated 3000 cpm). Appropriate antibody dilutions were obtained by incubating serial dilutions of antiserum with the standard amount of labeled ferritin in the assay procedure described above. That dilution which precipitated 30%–50% of the labeled ferritin was selected.

The immune complexes formed by the reaction of antiferritin antibodies with the mixture of iodinated ferritin and added standard isoferritin populations were coprecipitated along with carrier IgG by the appropriate anti-IgG antiserum. With rabbit antiserum, 50 μg rabbit IgG in 25 μl RIA buffer were added. With the guinea pig antiserum, 25 μl of nonimmune guinea pig serum, diluted to 4% (v/v) in RIA buffer, were added as a source of carrier IgG. The amount of anti-IgG antiserum used in each system was that which gave maximum precipitation of the radioactive immune complex. In both cases, this corresponded to 50 μl of the commercial anti-IgG antiserum. After diluting with 1 ml of RIA buffer, the reaction mixture was centrifuged for 20 min at 8500 g. The supernatant was decanted and excess fluid removed by careful swabbing with a cotton-tipped applicator. This swabbing procedure was found to give more consistent results and lower backgrounds than washing the precipitate with RIA buffer. The precipitated radioactivity was

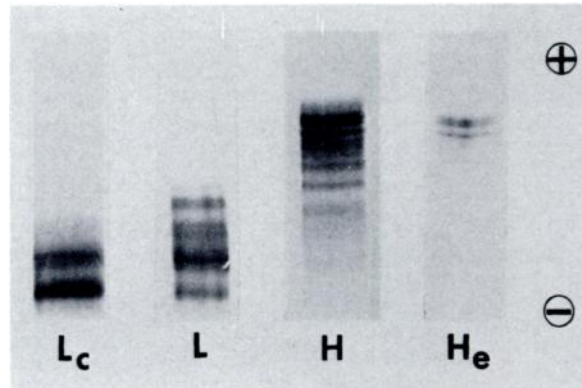


Fig. 1. Isoelectric focusing in 4% acrylamide gel, pH range 4–6, of ferritins from crystalline human liver (L_c), human liver (L), heart (H), and HeLa cells (He). After focusing, the gels were stained with Prussian blue.

counted in a Beckman Gamma 300 system. All assays were performed in duplicate or triplicate. Replicate cpm values usually agreed within 5%.

The amounts of ferritin in nanograms added to the assay were plotted against the ratio (B/B_0) of antibody-bound cpm in the presence of competing ferritin to antibody-bound cpm in the absence of competing ferritin. B/B_0 values in this assay are inversely related to the amount of the added ferritin and to its effectiveness in competing with the iodinated ferritin for binding to the antibodies.

RESULTS

In order to compare the immunologic relationships of human isoferritins, we selected several tissue ferritins whose isoferritin profiles represented different parts of the human ferritin spectrum. Figure 1 shows the isoferritin profiles of these ferritin preparations as displayed by gel electrofocusing. Unfractionated liver ferritin separated into multiple isoferritins which focused in the pH range 5.2–5.7, in agreement with previous analyses.^{21,22} The crystallized ferritin, however, had a less complex pattern, consisting largely of the two more basic isoferritins (pH 5.4–5.7), indicating a preferential crystallization of these forms. The heart ferritin had a more acidic pH range than the liver ferritin, although it did contain some of the more acidic liver isoferritins. Most of the heart isoferritins were found in the range pH 4.8–5.4. The HeLa ferritin had the most restricted spectrum of these tissue ferritins and, in agreement with previous results,¹⁸ corresponded to the most acidic of the heart isoferritins banding in the pH range 4.8–5.0.

These isoferritin profiles reflected the subunit composition of the ferritins. The most basic isoferritins consisted predominantly of the HL-type subunit, while the more acidic isoferritins contained progressively more of the H-type subunit. The relative percentage of the H and HL subunits in the ferritins was HeLa, H 70%, HL 30%; heart, H 40%, HL 60%; and liver, H 10%, HL 90%.¹⁷ Crystalline liver ferritin contained >90% HL and the natural apoferritin >98% HL. No L subunits were detected in these experiments since the samples were reduced prior to electrophoresis.²⁴

Previous studies^{18,21,22,27} have shown that all human tissue ferritins so far examined, including all those in the present study, give lines of apparent immunologic identity when tested in double diffusion against antibodies to normal liver ferritin. However, when the precipitation is examined quantitatively (Fig.

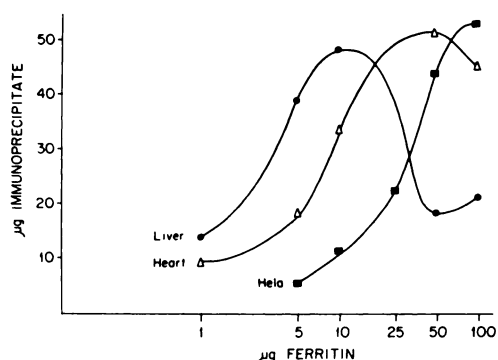


Fig. 2. Precipitin curves for rabbit anti-human liver ferritin IgG tested against ferritins from human liver, heart, and HeLa cells. Each tube contained 10 μ l IgG (15 mg/ml) in a total volume of 0.2 ml, and was incubated for 1 hr at 37°C, then 18 hr at 0°C. The samples were spun in a Brinkman microfuge and the precipitates washed four times before determining their protein content by the Lowry method.

2), liver ferritin is more reactive with anti-liver ferritin antibodies than are either heart or HeLa ferritins. Although the maximum amount of protein precipitated with all three ferritins is similar, significantly larger amounts of heart and HeLa ferritins are required to give the same level of precipitation as the liver ferritins. The reactivities of the different tissue ferritins appear to correlate with their content of HL subunit, indicating that the antibodies in the rabbit antiserum might be directed primarily against HL subunit determinants.

This specificity of the anti-liver ferritin antibodies also results in substantial differences in the apparent levels of different tissue ferritin populations in RIA procedures. In the assay used here, various isoferritin populations compete with ~ 1 ng 125 I-liver ferritin for a limiting amount of anti-liver ferritin antibodies. It can be seen in Fig. 3 that the apparent reactivities of the various ferritins are related to their content of the HL subunit, i.e., natural apoferritin > liver > heart > HeLa. For example, a reduction in B/B_0 to 0.7 would require the following amounts of the various ferritin preparations: 0.65 ng natural apoferritin (> 98% HL), 1.6 ng liver (90% HL), 7.5 ng heart (60% HL), and 13 ng HeLa (30% HL).

The opposite situation exists with antibodies whose specificity is directed

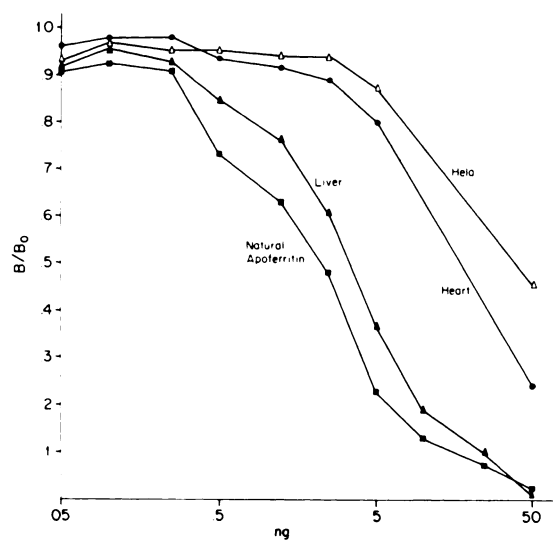
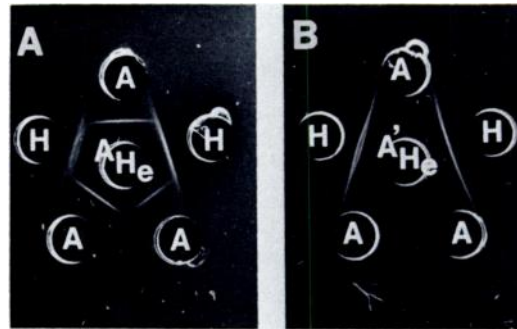


Fig. 3. Radioimmunoassay standard curves using 1:200,000 dilution of rabbit anti-human liver ferritin antiserum plus 1 ng 125 I-labeled liver ferritins and known amounts of various other human tissue ferritins including HeLa (Δ — Δ), heart (\bullet — \bullet), liver (Δ — Δ), and natural apoferritin (\blacksquare — \blacksquare). Methodologic details are described in the text.

Fig. 4. Ouchterlony double diffusion patterns showing heart ferritin (H) and natural liver apoferritin (A) reacted against (left-hand side) the guinea pig anti-HeLa ferritin antiserum and (right-hand side) the same serum after absorption with 20 μ g natural liver apoferritin/50 μ l serum.



toward the H subunit. We have previously shown that such antibodies can be obtained by raising antibodies to HeLa ferritin in guinea pigs and subsequently removing those antibodies directed against HL subunit determinants by suitable absorption.¹⁸ Figure 4A shows that the unabsorbed antiserum can distinguish between heart ferritin, which has approximately equal amounts of the H and HL subunits, and natural apoferritin, which consists almost entirely of the HL subunit. The spur of the precipitin line from the heart ferritin over that of the natural apoferritin indicates the presence of at least two populations of antibodies, one of which recognizes determinants common to both ferritin preparations, and another which recognizes determinants in heart ferritin but not in natural apoferritin. This antiserum has been made specific for H subunit determinants by absorbing with increasing amounts of natural liver apoferritin (Fig. 4B). This result implies a selective removal of antibodies reacting to the common determinants in both preparations, but not of those recognizing determinants unique to heart ferritin.

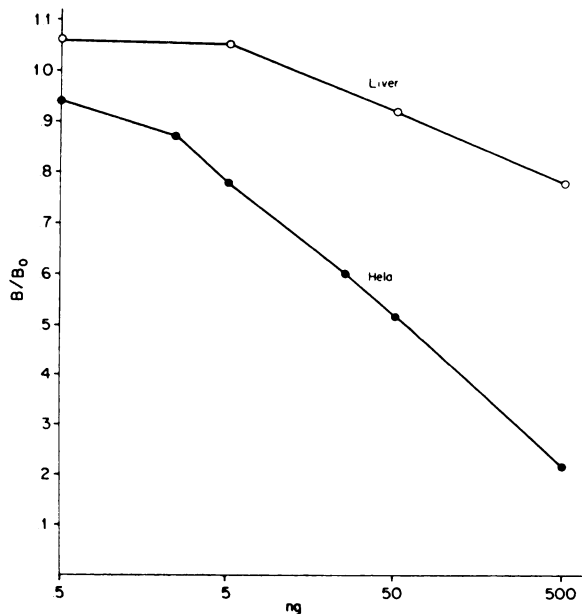


Fig. 5. Radioimmunoassay standard curves using 1:1,000 dilution of absorbed guinea pig anti-HeLa ferritin antiserum plus 10 ng ¹²⁵I-labeled HeLa ferritin and known amounts of human liver (○—○) and HeLa (●—●) ferritins.

As might be expected, this absorbed antiserum can be used in an RIA with ^{125}I -HeLa ferritin to discriminate between ferritin populations on the basis of their content of the H subunit (Fig. 5). In this assay a reduction of the B/B_0 ratio to 0.8 requires only 4.5 ng of HeLa ferritin (70% H), but 350 ng of liver ferritin (10% H).

DISCUSSION

Our results provide a structural basis for the observed differences in immunologic reactivities of different ferritin populations in RIA.^{10,20} The assays described here should be helpful clinically in providing qualitative as well as quantitative information about serum ferritin. Our results demonstrate that the apparent level of serum ferritin in RIA depends on four factors: (1) the serum isoferritin population, (2) the specificity of the anti-ferritin antiserum, (3) the character of the iodinated ferritin, and (4) the type of ferritin used as standards. The extent of the differences reported here among various isoferritin populations suggests that the levels of serum ferritin previously reported in many cases may require reevaluation. Most RIAs presently in use are standardized on a mixture of liver or spleen isoferritins. If, as now seems likely, much of the serum ferritin in normals or iron overload consists largely of the most basic isoferritins,¹⁷ it is probable that many assays may have considerably overestimated the actual level of serum ferritin (see Fig. 3). If the serum ferritin composition is similar to the whole spectrum of liver ferritin, as may be the case in diseases involving liver damage, the values found would be good estimates. On the other hand, if the serum ferritin consists of the more acidic, nonhepatic forms found in some cancers,^{14,28} which have only low reactivities with liver ferritin antibodies, the actual values might be underestimated by as much as two orders of magnitude. Consequently, it would be of considerable interest to examine cancer sera with an assay specifically tailored for these isoferritins. This approach might not only add a valuable dimension of specificity but, by selectively enhancing the sensitivity of the assay for these forms, considerably improve its usefulness in the serodiagnosis of some cancers. This type of assay might also find application in serodiagnosis of other pathologic states involving damage to tissues such as heart, kidney, and pancreas, whose ferritins contain higher proportions of the H subunit. Although information on the type as well as amount of ferritin in a sample might be obtained by using both RIAs described here, the heterogeneity in immunologic reactions involving complex mixtures of antibodies and multiple antigenic sites²⁹ precludes precise assignment of subunit ratios.

It should be mentioned that we have experienced considerable difficulty in iodinating HeLa ferritin to the same specific radioactivity as liver ferritin. While liver ferritin is easily iodinated by the standard³⁰ chloramine T method, either HeLa ferritin is not labeled or its immunological identity is destroyed. Although we have obtained adequate labeling by reducing the temperature and chloramine T concentration, the specific activity and immunologic reactivity between batches are highly variable. In addition, the iodinated HeLa ferritin preparations tend to form aggregates which are removed by centrifugation before use. This aggregation may also account for B/B_0 values in excess

of I at low levels of added liver ferritin (Fig. 5). Niederer³¹ has discussed this type of phenomena in other RIAs. Preliminary attempts at iodinating HeLa ferritin with the Bolton-Hunter³² reagent (New England Nuclear, Boston), which reacts with free amino groups, have been unsuccessful. Consequently, it may be better to construct an RIA specific for the H subunit by iodinating the antibody rather than the antigen.

Finally, our results clearly demonstrate the need for standardization of assays. In view of the variability of isoferritin populations in ferritin preparations from the same tissues of different individuals,³³ we suggest, for clinical studies of iron status, that crystalline human liver ferritin be used as immunizing antigen, labeled antigen, and reference standard. This ferritin is relatively easy to prepare and by virtue of its crystallization has a consistent isoferritin population whose subunit composition is similar to that commonly found in serum ferritin.

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