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Serum and Liver Isoferritin Differences: Evidence That Serum Ferritin May Be Underestimated in Hyperferritinemia (40570)

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Serum ferritin measurement has become a clinically useful, noninvasive indicator of iron stores in patients with suspected disorders of iron metabolism. However, serum ferritin is a heterogeneous pool of isoferritins (1, 2). These isoferritins are hybrid molecules arising from various combinations of two or more different subunit types. This accounts for the immunologic differences observed among various tissue and serum ferritins (2).

All radioassays for serum ferritin use ferritin extracted from liver or spleen as the reference standard and as the immunizing antigen to raise antiferritin antibody. It is, therefore, likely that these assays might not measure the true value of serum ferritin. Findings to support this hypothesis have been observed both with immunoradiometric assays (IRMA) (3, 4) and radioimmunoassays (2, 5).

In the present study we report additional evidence of the immunologic nonidentity of serum and organ isoferritins. Further, we propose that IRMA may actually underestimate the true value of serum ferritin in patients with various conditions of hyperferritinemia, including idiopathic hemochromatosis (IHC), liver disease, transfusional iron overload, cancer, leukemia, and chronic inflammation.

Materials and methods. For preparation of the human liver ferritin, the rabbit antihuman ferritin antibody and the purified ^{125}I-labeled antiferritin, the methods described by Ryan *et al.* (6) were used. We performed the IRMA as originally described by Miles *et al.* (7) and later modified by ourselves (8).

Preparation of liver ferritin. We extracted ferritin from human liver obtained at autopsy on subjects who died of aregenerative anemia and who had received multiple blood transfusions. Isolation of ferritin was carried out by cadmium sulfate precipitation of heated liver homogenate according to the method described by Mazur and Shorr (9). The purity of ferritin was checked by polyacrylamide gel electrophoresis as described previously (6) in which protein and iron stains of the gels show identity.

Purification of labeled antibody (Ab).* Antibody was raised against human liver ferritin in rabbits. This antibody was radioiodinated using aminocellulose as the immunoadsorbent, and the Ab* dissociated from its ferritin complex with a discontinuous HCl gradient (6). Three peaks of Ab* were obtained. The highest affinity Ab* was found in the pH 3.0 and 2.5 peaks (peaks II and III, respectively) (Fig. 1). Both peaks were used separately in our column experiments. When used in the IRMA, both Ab* peaks gave identical ferritin concentration values with unknown sera. In practice, these Ab* peaks are pooled for use in the assay. Following iodination, we further purified the Ab* by passage through a 30-cm column of agarose (Biogel A 0.5 m, exclusion limit 500,000 M_r). In the IRMA, the maximum efficiency of binding of this antibody to excess of liver or serum ferritin varied between iodinations from 56 to 85% in peaks II and III.

Quantitation of ferritin–antiferritin complex by gel filtration. In our experiments, mixtures of ferritin and labeled antiferritin were eluted through an agarose column to resolve ferritin–antiferritin complexes from unreacted labeled antiferritin. A fixed quantity of 100 ng liver or serum ferritin (determined by IRMA) was mixed with a predetermined amount of purified Ab*. We selected an amount of antibody that gave a linear relationship between the percentage Ab* bound and serial dilutions of liver ferritin over the range 200, 100, and 50 ng/ml (Fig. 2) using the column method to be described.

Ferritin and antiferritin were reacted in a barbital buffer containing bovine serum albumin (BSA buffer) which was made up as follows: To 7.5 g/liter sodium barbital (pH adjusted to 7.4 with 0.94 ml/liter concentrated hydrochloric acid) were added 4.5 g

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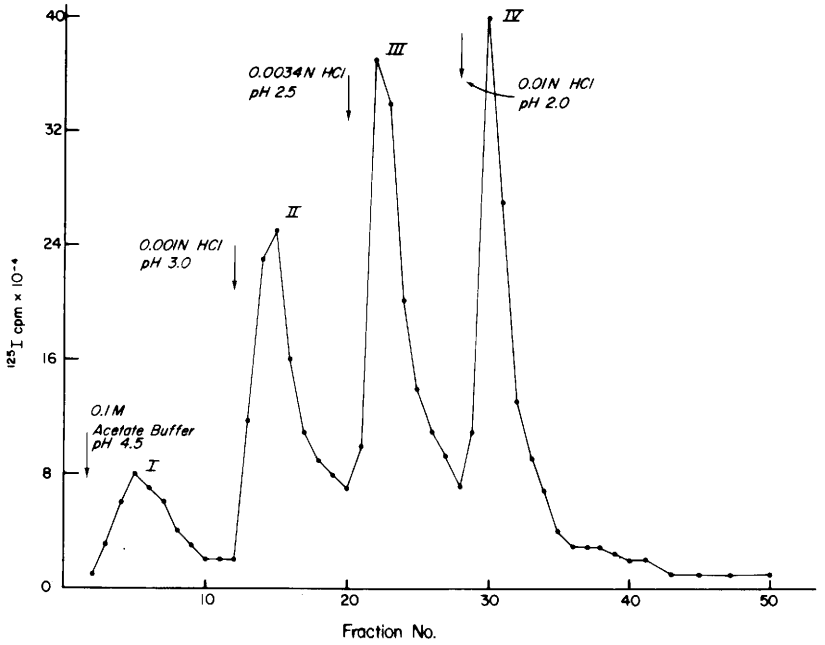

FIG. 1. Acid elution profile with a discontinuous HCl gradient of ^{125}I-labeled rabbit antihuman ferritin antibody (Ab*) obtained during purification. Immunoadsorbent containing the ferritin–antiferritin complex is applied to a Biogel P-2 column (6).

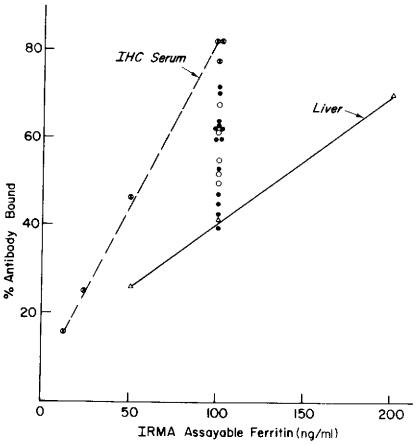

FIG. 2. Amount of ferritin antigen detected expressed as percentage Ab* bound to antigen measured with the Biogel column method: Linear dose–responses are obtained when serial dilutions of IHC serum (⊗– – –⊗) or liver ferritin (△– – –△) were reacted with Ab*. This was used to select the amount of antibody for the experiments carried out on 100 ng assayable liver or serum ferritin. Also shown are the percentages of Ab* bound when incubated with serum, containing 100 ng assayable ferritin, taken from patients with untreated IHC (⊗), partially treated IHC (○), and hyperferritinemia not due to IHC (●). These results are described below.

sodium chloride, 1.0 g BSA (Sigma fraction V), and 0.1 g sodium azide. The total volume of the reaction mixture was 1 ml and the reaction was carried out in a siliconized glass tube rotated for 1 hr at room temperature. After incubation 0.7 ml of the mixture was loaded on a 30-cm agarose column (Biogel A 0.5 m, 200–400 mesh), and 1-ml fractions were eluted using the BSA, pH 7.4 buffer. Duplicate tests were carried out on each specimen, and reproducibility was excellent.

All sera were heated before use to 60–65°C for 5 min then centrifuged at 400 g for 5 min. Heating serum improved recovery of total radioactivity loaded on the column from 40–60% to 90–100%. Ferritin protein withstands heat denaturation up to temperatures as high as 80°C for 10 min (10) and liver ferritin is subjected to heating during its purification. Heating serum in the manner described did not have any effect on the IRMA value.

When 100 ng extracted human liver ferritin was mixed with the Ab*, heated normal rabbit serum or ferritin-deficient human serum was added to the reaction mixture in amounts equivalent to that present when serum was being tested. The total volume of reaction

mixture remained the same.

Clinical material. Serum from a total of 21 patients with hyperferritinemia were included in this study. The clinical diagnoses in these patients were as follows: IHC (3 untreated, 5 undergoing phlebotomy treatment), liver disease (4), transfusional iron overload (4), cancer (1), leukemia (2), and chronic inflammation (2). Measured by the IRMA the range of serum ferritin concentrations was 350 to 5500 ng/ml.

Results. When Ab* alone was passed through the agarose column, we obtained a single peak of radioactivity. When this Ab* was mixed with ferritin-deficient serum, again only one peak was obtained (Fig. 3A). However, when a source of ferritin was added, an earlier peak representing Ag–Ab* complex was eluted in the void volume (Figs. 3B and C).

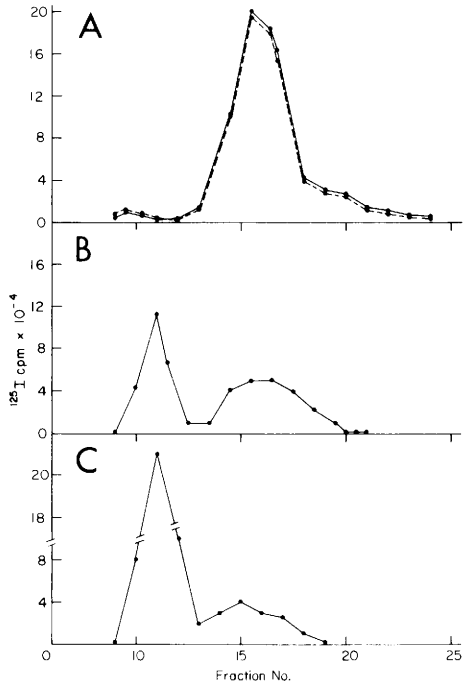

FIG. 3. (A) Single peak of unbound Ab* obtained when Ab* alone (●– – –●) or Ab* mixed with ferritin-deficient serum (●——●) is passed through a Biogel A 0.5-m column. (B) An earlier peak representing antigen–antibody complex is obtained when liver ferritin is added to Ab*. This represents 41% of the total radioactivity added as radiolabeled antibody. (C) The same amount (by IRMA) of serum ferritin from a patient with IHC added to Ab*. The antigen–antibody peak is increased to 81% of the total radioactivity added.

Figure 1 shows the typical HCl elution profile of Ab* that is obtained during iodination using aminocellulose as the immunoadsorbent. When purified peaks II or III Ab* was mixed with 100 ng of liver or serum ferritin from patients with hyperferritinemia then eluted through the agarose column, we noted clear differences between liver and serum ferritin in the amount of Ag–Ab* complex formed (Fig. 4). With serum ferritin, there was a greater amount of Ab* in the antigen-bound peak than with equivalent assayable amounts of liver ferritin. The mean proportion of Ab* in the first peak (Ag–Ab* complex) expressed as a percentage of the total counts loaded on the column in four experiments using liver ferritin was 41% (range 40–42%). When the same quantity of ferritin, as determined by IRMA, from the serum of patients with untreated IHC was mixed with the Ab*, a mean of 81% (79, 81, and 82%) of the total load appeared in the first peak (three patients tested). We also studied sera from five patients with hyperferritinemia due to other causes. Most of the patients showed considerably higher Ag–Ab* complex peaks than with an equivalent amount of liver ferritin (Fig. 2). The amount of antigen detected in the 5 IHC sera ranged

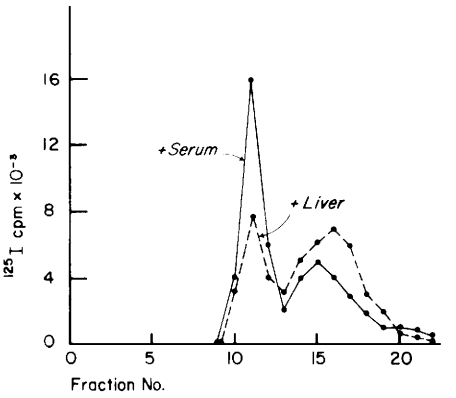

FIG. 4. Elution of ^{125}I-labeled rabbit antihuman ferritin antibody reacted with liver or serum ferritin (see text) using BSA buffer, pH 7.4, through a Biogel A 0.5-m column. The first peak represents antigen–antibody complex, the second peak unbound antibody. More ferritin antigen is detected with serum ferritin than with an equal amount (by IRMA) of liver ferritin.

from 54 to 67% and in the 13 other hyperferritinemia sera from 39 to 72%. The mean value for all sera was 60.4%.

Discussion. Several studies have reported the heterogeneous isoferritin profile of various tissues as well as serum in health and disease (1, 2, 11–14). It is now apparent that in IHC as well as in other states of iron overload, the serum isoferritin profile is altered. Furthermore, the profile of serum ferritin in IHC changes during phlebotomy therapy (15, 16), the normal distribution of tissue isoferritins eventually being restored (17). Hazard and Drysdale (5) using a specific Hela-type radioimmunoassay (RIA) found a preponderance of the acidic "Hela-type" isoferritins in all sera from 15 patients with various solid tumors. They showed that the standard "liver-type" assay grossly underestimates the true serum value in patients with cancer.

Using a different approach, our findings support those of Hazard and Drysdale. Moreover, our results suggest that the standard IRMA frequently may underestimate ferritin concentration in the majority of patients with hyperferritinemia, not only those with cancer. Using a gel filtration technique, we found that more immunoreactive ferritin is detected in serum than in an equivalent amount (by IRMA) of liver ferritin. For reasons that are not clear, the conventional IRMA fails to detect a proportion of this immunoreactive ferritin in serum.

Halliday *et al.* (15) suggest that the rapid, early fall in serum ferritin concentration that they observed during phlebotomy treatment for IHC, out of proportion to the decrease in iron stores, might be explained by a change in isoferritin pattern. This could also explain the findings we report here of higher antigen detection, using the column technique, in untreated IHC than in our IHC patients who had already started their phlebotomy program.

Our findings using a simple gel-filtration technique, suggest that IRMA may underestimate the true serum ferritin level in many cases of hyperferritinemia. Proportional dose–response in the IRMA may give clinically useful information on ferritin values, but isoferritin differences may vitiate accurate quantitation of the serum ferritin and may tend to underestimate the true mass of ferritin protein present.

Summary. We have used gel filtration chromatography to compare relative antibody affinities of liver ferritin and serum ferritin from patients with idiopathic hemochromatosis and other causes of hyperferritinemia such as liver disease, transfusional iron overload, and cancer. When equal amounts of either liver or serum ferritin were mixed with ^{125}I-labeled antiferritin antibody then eluted through an agarose column, we obtained higher antigen–antibody complex recovery with the serum ferritin than liver ferritin. This was more marked in patients with untreated, idiopathic hemochromatosis. Our findings provide additional evidence of different reactivity between serum isoferritins and an antibody prepared against organ-specific ferritin. Further, this suggests that the immunoradiometric assay may underestimate serum ferritin concentration in patients with high ferritin values.

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