Quantitation of Ferritin Iron in Plasma, an Explanation for Non-Transferrin Iron

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In 33 patients with thalassemia and idiopathic hemochromatosis, plasma ferritin protein levels ranged from 36 to 5,850 μg/L. The iron content of this ferritin as determined by immunoprecipitation ranged from undetectable amounts to 507 μg/L. The mean iron content of ferritin protein in those and other subjects with plasma ferritin concentrations of over 1,000 was 6.8% ± 2.7%. Plasma transferrin was usually saturated with iron in patients with measurable ferritin iron, but exceptions occurred. In studies using electrophoretic separation, it was shown that some ferritin iron moved to transferrin during in vitro incubation, whereas exchange in the opposite direction was extremely limited. Because some plasma ferritin iron was measured by the standard colorimetric plasma iron determination, these observations (a) indicate that plasma ferritin contains a significant amount of iron (b) indicate that a significant proportion of nontransferrin iron in individuals with nontransferrin iron as detected by standard plasma iron and total iron-binding capacity measurements is due to the presence of ferritin, and (c) suggest that large amounts of ferritin iron may affect the saturation of plasma transferrin.

Virtually all plasma iron in the normal individual is bound to the iron transport protein transferrin. In subjects with idiopathic hemochromatosis and thalassemia, however, there is convincing evidence that nontransferrin iron is present in significant amounts. This has led to the speculation that such iron may be responsible for tissue damage. To investigate the nature of this nontransferrin iron, we have developed a quantitative immunoprecipitation technique for measuring the ferritin iron content and have examined the possibility that the nontransferrin iron in patients with thalassemia is ferritin.

Materials and Methods

Preparation of the plasma and serum samples. Blood was drawn in iron-free disposable syringes into heparin or without anticoagulant from three groups of subjects. Samples from patients with thalassemia came from the hematologic clinic of the Siriraj Hospital, Bangkok, Thailand. These patients had β-thalassemia/hemoglobin E disease and hemoglobin II disease. Six of the β-thalassemia/hemoglobin E patients had received from four to 15 red cell transfusions, and one of the hemoglobin H patients had had four transfusions, but none had been given in the 3 months preceding blood collection. There were no signs or symptoms of infection at the time. Blood from patients with idiopathic hemochromatosis at different stages of iron depletion was drawn by phlebotomy in the Hematology Research Unit, University of Washington, Seattle. Blood was also drawn from fasting, healthy laboratory personnel who had been shown by standard tests to have normal iron status.

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Submitted May 29, 1987; accepted December 14, 1987.

Supported in part by US Public Health Research Grant HL34408 from the National Heart, Lung, and Blood Institute; by National Institutes of Health Grant HL06242; and by the International Atomic Energy Agency Fellowship Program of the National Research Council, National Academy of Sciences.

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Plasma or serum was initially characterized by determining plasma iron levels, total iron-binding capacity (TIBC), and the ferritin content. Additional studies were carried out on fresh plasma or after storage at −20°C. When stored plasma was used, 1 mol/L sodium bicarbonate solution (50 μL/mL plasma) was added and the plasma then incubated at 37°C for 15 minutes to provide sufficient bicarbonate ions to facilitate iron binding to the transferrin molecule. In some studies on normal plasma, the transferrin iron saturation was adjusted in vitro to higher levels by the addition of ferrous ammonium sulfate.

Plasma ferritin iron determination. Antibody against human ferritin was produced in rabbits by the injection of a highly purified liver ferritin. Rabbit antisera of high titer was purified by precipitation with 25% ammonium sulfate. The precipitate was washed twice with 1.75 mol/L ammonium sulfate and then dialyzed twice for 12 hours against distilled water at 4°C, for 24 hours against 0.05 mol/L sodium acetate−0.21 mol/L HAC buffer, pH 5, for 24 hours against distilled water, and for 24 hours against acetate buffer, pH 5. The dialysate was centrifuged and the supernatant transferred to a diethyl aminoethyl (DEAE)-Sephacel column equilibrated with 0.071 mol/L acetate buffer, pH 5. The first protein peak (void volume) eluted with acetate buffer was collected and pooled, adjusted to pH 8.3 with 1 mol/L Tris base, and centrifuged. The supernatant was applied to a DEAE-Sephacel column equilibrated with 0.05 mol/L Tris/HCl, pH 8.3. The IgG was eluted by use of a gradient, 0.05 mol/L Tris/HCl, pH 8.3, to 0.5 mol/L Tris/HCl, pH 8.3. The first protein peak was IgG. This double-column technique eliminates residual transferrin from the IgG preparation. The IgG-containing peak was concentrated to 10 to 20 mg IgG/mL by Amicon filtration with a PM 10 membrane (Amicon Corp, Danvers, MA) and washed with bicarbonate saline that had been freed of iron by chelax treatment (Bio-Rad Laboratories, Richmond, CA). Concentrated IgG was coupled to Affi-gel 10 (Bio-Rad) at a concentration of 40 to 60 mg IgG/mL of Affi-gel according to the procedure recommended by Bio-Rad. After coupling, the Affi-gel 10–Ab was washed several times with iron-free bicarbonate saline and resuspended in 4 vol bicarbonate saline.

To determine the plasma ferritin iron content, 0.2 mL Affi-gel–Ab suspension was added to 2 mL plasma. The solution was mixed overnight at 4°C and then centrifuged at 1,400 g for 30 minutes, after which the ferritin-depleted supernate was removed, checked for residual ferritin by an enzyme-linked immunosorbent assay technique, and then discarded. It was also checked for ferritin iron by using 59Fe-tagged ferritin. An amount of ferritin and ferritin iron <5% of the original value was considered satisfactory. The Affi-gel–Ab–ferritin complex was washed twice with iron-free HCO3-saline and centrifuged each time at 1,400 g for 30 minutes at 4°C. One milliliter of 3 N HCl was then added to the precipitate, mixed, and incubated overnight at 37°C, after which 250 μL of 20%
trichloracetic acid was added, mixed, and centrifuged for 20 minutes at 1,400 g. One milliliter of protein-free supernatant was transferred to an iron-free tube to which 1 mL of saturated Na acetate and 100 μL of 0.2% bathophenanthroline with 1% thioglycolic acid were added. After 20 minutes the amount of color was determined at 535 nm on a spectrophotometer. Appropriate blanks and standards were prepared, including an Affi-gel blank using either known low-ferritin plasma or nonimmune rabbit serum. The optical density (OD) of the Affi-gel in saline was 0.01 ± 0.002, and that of normal plasma (ferritin, 20 to 90 μg/L) was 0.015 ± 0.004. When iron was added to oversaturate the transferrin of normal plasma, the OD reading of iron in the precipitate was 0.015 ± 0.005. The OD of ferritin iron added to plasma to a concentration of 10 μg/L was 0.167.

Electrophoretic separation of ferritin and transferrin. In studies using either 55Fe-labeled transferrin or ferritin, plasma was incubated at 37°C for one to 72 hours and then separated by electrophoresis.15 The incubated mixture was applied to either 7.5% or 6% polyacrylamide gel columns. The vertical-disk polyacrylamide electrophoresis was performed at 80 V in 0.215 mol/L Tris-glycine buffer for 90 minutes and was followed with 160 V for either 180 or 270 minutes. The electrophoresis gel was then cut into 2-mm slices. The radioiron activity in each piece of gel was counted in Packard gamma counter (model 5330). The radioactive profile was plotted and the radioactivity in each peak determined as a percentage of the total activity. The radioiron-labeled ferritin used in these studies was prepared as described elsewhere.14

The plasma transferrin content was also measured by an immunoprecipitation technique. Bovine transferrin was similarly precipitated by a specific antibody and the iron content of the precipitate measured colorimetrically.9,11,12

Another way of evaluating the behavior of nontransferrin iron was by the addition of 1 mg bovine apotransferrin to 3 mL thalassemic plasma that was oversaturated by the standard plasma iron/TIBC measurements.12 The mixture was incubated at 37°C, and 0.3-mL samples were taken at intervals up to 24 hours. Individual samples were treated with antibovine transferrin antibody, and the iron content of the centrifuged precipitate was measured by a colorimetric procedure.12

The statistical significance of the differences observed between groups of animals was assessed by Student’s t tests with pooled or separated variances, as appropriate.

RESULTS

The immunoprecipitation technique used for quantitating ferritin iron showed a mean value of 5 ± 4 μg/L (1 SD) in normal individuals with plasma ferritin protein levels of 20 to 80 μg/dL. These values were validated by using 10 instead of 2 mL of plasma for immunoprecipitation.

In 18 patients with thalassemia whose plasma ferritin levels ranged from 36 to 5,850 μg/L, ferritin iron varied from undetectable amounts to 366 μg/L. In 15 patients with idiopathic hemochromatosis and ferritin values of 45 to 4,520, the plasma ferritin iron level varied from 12 to 507 μg/L. In general, the values for ferritin iron corresponded to the amount of ferritin present (Fig 1). The mean iron concentration of ferritin in seven thalassemic patients with values over 1,000 μg/L was 7.7% ± 3.1%, and in nine patients with hemochromatosis, it was 5.1% ± 1.7%. Above 1,000 μg/L ferritin protein, the overall concentration of iron in ferritin was 6.8% ± 2.7%. In 25 plasmas from these and other patients with ferritin protein values from 50 to 1,000 (mean, 225 ± 212 μg/L), there was a ferritin iron level of 16 ± 23 μg/L and an iron content of 5% ± 4%. In seven patients with ferritin protein values between 1,000 and 2,000, the iron content averaged 6.0% ± 3.5%. In five patients with ferritin values between 2,000 and 3,000, the iron content was 7.2% ± 1.6%, and in four patients with ferritin protein values above 3,000, the mean iron content was 7.7% ± 2.5%. There appeared to be a progressive increase in ferritin iron concentration as ferritin protein levels increased, but differences were not statistically significant.

The relation between transferrin saturation and ferritin iron in patients with thalassemia and idiopathic hemochromatosis is shown in Fig 2. For the most part, patients with significant amounts of ferritin iron had a saturation of greater than 100% as determined by the standard colorimetric measurement of plasma iron and TIBC. In one sample taken from a patient 15 days after a marrow transplant who showed pancytopenia characteristic of pretransplant therapy but no other complications, the transferrin saturation was 74%; ferritin protein level, 7.200 μg/L, and ferritin iron level, 546 μg/L. Of possible interest was the absence of measurable ferritin iron in three patients with chronic inflammation who had ferritin protein concentrations between 200 and 676 μg/L.
Therapidity of the initial exchange is shown in Fig 3. When transferrin was oversaturated as determined by standard measurements of plasma iron and iron-binding capacity. The iron content of the transferrin peak within one hour and 25% by 72 hours. The oversaturation suggested by standard plasma iron measurements by electrophoresis. On the other hand, radioiron-labeled ferritin showed appreciable relocation. When iron-labeled transferrin was incubated with plasma containing unsaturated transferrin, about 10% of the ferritin iron moved to the transferrin peak within one hour and 25% by 72 hours. The rapidity of the initial exchange is shown in Fig 3. When labeled ferritin was incubated with saturated transferrin, there was no transfer at one hour, but there was a 15% transfer at 72 hours.

The effect of ferritin iron on the standard measurement of plasma iron could be demonstrated by removal of transferrin iron. When transferrin was quantitatively precipitated by a specific antibody, the nonprecipitated supernatant of individuals with transferrin saturations of >100% showed measurable iron, whereas that of normal subjects did not. In six subjects with ferritin values from 900 to 9,000, the nonprecipitable iron concentration measured by the plasma iron method varied from 21 to 118 μg/L. This was consistent with the 6% to 7% iron content of ferritin because only about one third of the ferritin iron would be measured.15

**DISCUSSION**

The presence of nontransferrin iron is suggested when the plasma iron concentration is shown to exceed the measured TIBC.15 In marked iron overload the saturation is usually about 110% but may reach 140%. Because the colorimetric methods usually used are insensitive to heme iron,16 the excess iron is assumed to be some other form. Studies using added radioiron to detect nontransferrin iron14 can do no more than indicate the lack of radioiron binding, that transferrin is nearly or completely saturated.

Hershko and Rachmilewitz were the first to present convincing evidence that the nontransferrin iron was chelatable because it was found in an ultrafiltrate of plasma to which EDTA had been added.2,17 Another method developed by the same investigators involved the use of a DEAE-Sephadex A-50 catechol disulphonate column where the difference between plasma iron entering and leaving was assumed to represent nontransferrin iron.7 Although 95% to 100% of transferrin passed through the column and chelated ionic iron was held back, other compounds such as ferritin were retained.18 Thus the demonstration of retained iron in thalassemia by Hershko et al13 and Anuwatanakulchai et al6 when using this technique does not specify the form of this iron. More recently, Gutteridge et al19 have reported a method not requiring chelation. They found that in the presence of iron, DNA would be degraded as indicated by a change in the spectral properties of bleomycin. The plethora of patients with idiopathic hemochromatosis and saturated transferrin produced this reaction. The reaction did not occur when apotransferrin was added; however, Wagstaff et al20 using the same approach found that four of six patients with increased stores who showed the reaction did not have a saturated transferrin concentration. Because of contradictory results and questions concerning the nature of the nontransferrin iron, we chose to examine the possibility that this might be derived, at least in part, from plasma ferritin.

Ferritin is known to be present in plasma and to be considerably increased in iron overload and after damage to iron-rich tissues.21 It is partially measured by standard colorimetric methods for plasma iron.13 A method was developed whereby plasma was precipitated by a specific polyclonal antibody and its iron content measured. Earlier studies, although made difficult by the small amount of ferritin present, indicated that the iron content of plasma ferritin was between 2% and 7%, appreciably lower than the tissue ferritin iron content in the same patients.22 Our mean ferritin iron content of 7% was slightly higher but lower than the expected tissue ferritin iron value, which may reach about 20% in subjects with iron overload. Nevertheless, the amount of ferritin iron present in the patients studied was sufficient to explain the oversaturation suggested by standard plasma iron and binding capacity measurements.15 The similar iron content of ferritin in plasmas with ferritin concentrations ranging from 500 to 5,000 μg/L suggests little effect of the amount of storage iron in body tissues on the plasma ferritin iron content.

It is not possible at the present time to resolve apparent discrepancies between the present study and prior studies that have been interpreted to show "free iron" in the plasma. Indeed, both sources may be present, and the differences in results may depend on differences in the experimental subjects tested. We have demonstrated that some ferritin iron shifts in vitro to apotransferrin, but it would not be expected that appreciable amounts of ferritin iron would shift to EDTA in the concentration used in ultrafiltration studies. Further studies comparing methods in the same patients are needed. The difference may be important because the potential of free iron for creating tissue damage may be greater than that of ferritin-associated iron.
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