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Ferritin in Formed Blood Elements[1] (38539)

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Recently developed methods have shown that the storage iron protein, ferritin, is consistently present in circulating serum (1, 2). The level of plasma ferritin bears a close relationship to the size of body iron stores (3–6) and is also influenced by the functional state of the RE cell and hepatocyte (7). The present study examines the concentration of ferritin in the various cell fractions of blood in normal subjects and in individuals with disorders in iron balance.

Methods. Studies were performed on five normal male and five normal female volunteers between the ages of 15 and 50 yr. Their mean hematocrit was 42% (range 36–44), mean transferrin saturation 38% (range 33–48) and mean total iron binding capacity (TIBC) 335 μg/100 ml (range 312–361). Four patients with iron deficiency anemia had a mean hematocrit of 30% (range 24–32), hypochromic microcytic red cells, a mean transferrin saturation of 6% (range 4–7), and a mean TIBC of 471 μg/100 ml (range 420–541). Five patients with untreated hemochromatosis had transferrin saturations averaging 96% (range 93–98) and a liver biopsy showing a marked increase in parenchymal iron. Five patients with an acute bacterial infection had a mean oral temperature of 103°F (101–104°) a mean white cell count of 16,400/mm^3 (range 11,000–19,000), a mean hematocrit of 33% (range 31–34) and a mean serum iron of 34 μg/100 ml (range 22–198). Six additional patients were studied prior to and following a surgical procedure (menesectomy, vaginal hysterectomy, and laparotomy). The mean preoperative hematocrit in this group was 41% (range 35–50) mean transferrin saturation 45% (range 34–56) and mean TIBC 250 μg/100 ml (range 219–280). Measurements in three patients with a reticulocytosis between 16 and 26% included a patient with autoimmune hemolytic anemia, a patient with hereditary spherocytosis, and a patient with iron deficiency anemia responding to parenteral iron therapy.

Specific cellular fractions of blood were prepared on a gravity gradient (8). Heparinized blood in a volume of 7.5 ml was carefully layered on 3 ml Hypaque–Ficoll (10 parts 34% Hypaque [Hypaque sodium 50%, brand of sodium diatrizoate USC] and 24 parts 9% Ficoll [Pharmacia Fine Chemicals AB, Uppsala, Sweden]) adjusted to a specific gravity of 1077. After centrifuging at 400 *g* for 35 min, a *mononuclear layer* containing monocytes and lymphocytes was removed, washed twice in phosphate buffer (0.2 *M* phosphate in isotonic saline, pH 7.4), and diluted in phosphate buffer to a final volume of 1 ml. In normal subjects this fraction contained a mean of 8.6×10^6 cells/ml of which 96.4% were lymphocytes and monocytes. This fraction also contained a mean of 69.8×10^6 platelets/ml.

After removal of the mononuclear layer, a *granulocyte* fraction was obtained by suspending the remaining cells in 6 ml of isotonic saline containing 3% Dextran. After allowing red cells to sediment for 30 min, the granulocyte-rich supernatant was removed and mixed with 7 ml of 0.87% NH_4Cl to lyse the residual red cells. The sample was then centrifuged at 220 *g* for 5 min, the cell sediment was washed once in phosphate buffer and made to a final volume of 1 ml. In normal subjects, this fraction contained a mean of 10.2×10^6 cells of which 98.1% were granulocytes. Platelets were not detected in this fraction.

After removing the granulocytes, the red cells remaining after Dextran sedimentation

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were suspended in 10 ml phosphate buffer and centrifuged at 1500 *g* for 15 min. The supernatant and upper third of the red cell column were removed and the remaining cells were suspended in 10 ml phosphate buffer. This procedure was repeated twice, each time removing the upper third of the red cell column. The final red cell fraction was diluted with phosphate buffer to give a hemoglobin concentration of approximately 13 g/100 ml. The fraction contained a mean of 0.17 $\times$ 10^6 granulocytes/ml. Mononuclear cells and platelets were not detected and there was less than one reticulocyte/1000 red cells.

A *reticulocyte-rich fraction* was prepared from blood of patients with increased erythropoiesis by first removing mononuclear cells and granulocytes as described above. The remaining red cells were washed once in 10 ml phosphate buffer, resuspended in twice the original volume of isotonic saline, layered on 10 ml of 27% Dextran and centrifuged at 15,000 *g* for 15 min (9). The upper layer containing the less dense reticulocytes was removed, the cells were washed twice in 10 ml phosphate buffer, and diluted in phosphate buffer to a hemoglobin concentration between 10 and 14 g/100 ml. *Platelet-rich plasma* was obtained as described by Tangen *et al.* (10) by centrifuging 10 ml heparinized blood at 300 *g* for 10 min. The platelets were then extracted from other plasma constituents by passing 2.5 ml of plasma through a 14 $\times$ 1 cm Sepharose 2B column. The platelets contained in the initial 1 ml void volume were collected and saved. The mean platelet count of this preparation in normal subjects was 295 $\times$ 10^6 platelets/ml. Leukocytes and granulocytes were not detected in this fraction.

Ferritin was released from the various cell fractions by ultrasonication for 30 sec using a Biosonic ultrasonicator. Examination of the sediment following ultrasonication showed only cellular debris in contrast with the many intact cells seen with lysis by repeated freeze–thawing or heating to 70° for 10 min. Purified ferritin was not altered by the ultrasonication procedure as evidenced by ferritin values averaging 99 ng/ml (range 98–104) in 6 determinations following ultrasonication of a ferritin solution containing 100 ng/ml.

Following ultrasonication and centrifugation, ferritin determinations on the supernatant were performed by a method previously described (2) with the exception that standards were prepared in diluent containing no added serum. Serial dilutions of each of the isolated cell fractions ranging from 1:10 to 1:250 revealed no systematic errors in the assay. Studies in which purified ferritin was added to each of the cell fractions in amounts varying from 200 to 300 ng/ml showed recoveries ranging from 90 to 119%. Comparisons were also made of the ferritin concentration in the supernatant of the various cell fractions before and following ultrasonication. In the case of granulocytes and erythrocytes, the value prior to ultrasonication was less than 1% of the value following ultrasonication. In contrast, the supernatant of the mononuclear fraction prior to ultrasonication contained an average of 9% (range 4–20) of the value following ultrasonication. Repeated washings of this cell fraction was accompanied by a progressive fall in the cell counts, indicating that this phenomenon was due to cell breakage. Thus, the ferritin content of the supernatant of the mononuclear fraction, before ultrasonication, was routinely measured and subtracted from the value after ultrasonication.

Erythrocyte and mononuclear fractions were contaminated with granulocytes and platelets respectively. For the erythrocyte fraction a correction was applied for contaminating granulocytes based on the number of granulocytes present and their ferritin content as determined for measurements on the purified granulocyte fraction. In 10 normal subjects, the correction averaged 6% (range 1–11) of the ferritin concentration in the erythrocyte fraction. In studies in 10 normal subjects, an attempt was made to measure the concentration of platelet ferritin. In purified platelet fractions containing 151–454 $\times$ 10^6 cells/ml, the mean ferritin value was 3 ng/ml which is equivalent to a contribution of 2 ng/ml whole blood or less than 2% of the ferritin concentration in whole blood. Platelets, therefore, did not contribute significantly to either the ferritin concentration of whole blood or the mono-

nuclear fraction at the level of platelet contamination observed.

For the granulocyte and mononuclear (lymphocyte and monocyte) fractions, the concentration of ferritin per 10^6 cells was converted to concentration per milliliter of packed cells assuming a cell volume of 400 μ^3_m for both granulocytes and mononuclear cells. Erythrocyte ferritin was initially related to the hemoglobin concentration of the hemolysate and then converted to concentration/ml of packed red cells based on the hemoglobin concentration of whole blood and the hematocrit. The contribution of each of the fractions to the ferritin concentration of 1 ml of whole blood was calculated on the basis of the number of these cells originally present. This value was compared with direct measurements of ferritin concentration in whole blood which had been ultrasonicated and centrifuged as described above.

Measurements of the hemoglobin, hematocrit and white cell counts were performed using standard techniques. Serum iron was measured by the method recommended by the International Committee for the Standardization in Hematology (11) and the TIBC determined by a radioactive magnesium carbonate technique (12). As with plasma ferritin (5), ferritin values for the formed elements were lognormally distributed. Statistical analysis was therefore performed on the logarithmic scale and the results retransformed as antilogarithms to recover the original units of measurement.

Results. The ferritin concentration in serum, whole blood and various cellular fractions in 10 normal subjects are shown in Fig. 1a. The mean serum ferritin was 44 ng/ml with a range from 21 to 84 ng/ml. The mean whole blood ferritin in these subjects was 108 ng/ml reflecting the higher ferritin concentration in the formed blood elements. Granulocyte ferritin in normal subjects averaged 24,700 ng/ml packed cells and was remarkably similar to the mean of 23,100 ng/ml for the mononuclear cell fraction. The mean ferritin concentration of 100 ng/ml in mature erythrocytes was only slightly higher than in serum.

In Fig. 1b is illustrated the changes in ferritin concentration observed in patients with iron deficiency, iron overload and infection. Serum ferritin levels were consistent with values previously reported in these disorders (7). Thus, in the iron deficient subjects, serum ferritin averaged 3 ng/ml with a range of 1–8. In iron overload the serum ferritin averaged 1200 ng/ml whereas with infection an intermediate elevation averaging 310 ng/ml was observed. This pattern was reflected in ferritin concentrations of whole blood which averaged 30 ng/ml in iron deficiency and 2900 ng/ml in iron overload. In contrast with serum levels, some overlap in whole blood ferritin concentration was observed between subjects with iron deficiency and acute inflammation (mean value 1257 mg/ml). The ferritin concentration in granulocytes was decreased with iron deficiency to 4600 ng/ml and was significantly increased with inflammation to a mean of 124,300 ng/ml. In subjects with iron overload, the mean concentration of granulocyte ferritin was almost identical to that in normal subjects. In the mononuclear fraction of iron deficient subjects, the mean of 23,100 ng/ml was only slightly lower than in normal subjects, whereas an increase to 55,600 ng/ml and to 146,000 ng/ml was observed in iron overload and inflammation respectively. The ferritin concentration in mature erythrocytes showed much less variation in these disorders than the serum ferritin. Thus, with iron deficiency, erythrocyte ferritin was decreased to a mean of 46 ng/ml, and increased to means of 665 and 1200 ng/ml with iron overload and inflammation respectively.

In Table I is shown the contribution of the various cell fractions to the ferritin concentration of 1 ml whole blood. Comparisons also are made in this table between the whole blood ferritin calculated from analysis of independent cell fractions and that determined by direct measurement. In normal subjects the contribution of the serum, granulocytes, mononuclear cells and erythrocytes was roughly equal. In iron overload, the marked increase in whole blood ferritin was largely due to the elevation in serum ferritin. A similar finding was seen in iron deficiency anemia where the reduc-

TABLE I. FERRITIN IN SERUM AND FORMED BLOOD ELEMENTS IN NORMAL SUBJECTS AND PATIENTS WITH DISORDERS IN IRON METABOLISM.

Group	Number	Ferritin (ng/ml whole blood)[a] Serum	Granulocyte	Mononuclear	Erythrocytes	Whole Blood Indirect[b]	Whole Blood Direct
Normal	10	25 (16–40)	37 (31–44)	19 (12–30)	42 (33–51)	128 (109–152)	108 (86–137)
Hemochromatosis	5	1131 (679–1886)	28 (13–53)	45 (24–83)	357 (180–709)	1653 (1103–2478)	2912 (2139–3963)
Iron deficiency	4	1.5 (0.5–4)	8 (4–16)	16 (9–27)	13 (9–23)	42 (30–59)	31 (21–44)
Inflammation	5	213 (104–437)	650 (373–1135)	130 (64–261)	152 (73–317)	1127 (621–3426)	1256 (693–2279)

[a] Values listed are geometric means. The limits of ± 1 SD are given in parenthesis.
[b] Total calculated indirectly from analysis of the separated fractions.

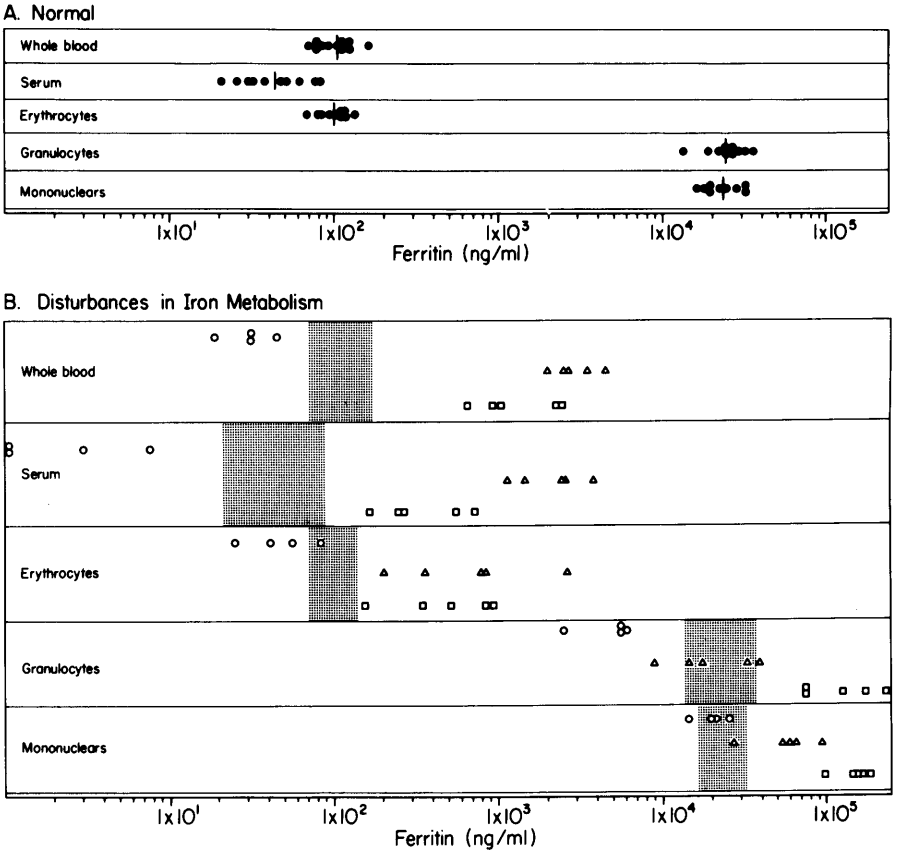

FIG. 1. Ferritin in whole blood and its components. Analysis was carried out in 10 normal subjects (A) and in subjects with disorders in iron metabolism (B). Subjects with iron deficiency are portrayed by open circles, with iron overload by triangles, and inflammation by squares. The normal range is shown by shaded areas. Ferritin concentration is expressed as nanograms per milliliter of the isolated fraction.

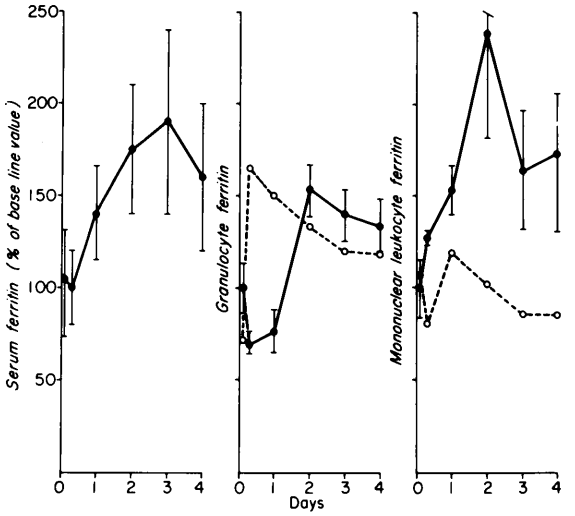

FIG. 2. Changes in ferritin concentration of cellular components of blood following surgery. Results, expressed as % change from the preoperative value, are shown as solid lines. Vertical lines represent plus or minus one standard error. Interrupted lines represent % change in granulocyte and mononuclear cell counts.

tion in whole blood ferritin was again the result of a marked decrease in the serum compartment. The increase in whole blood ferritin observed with inflammation was the result of increase in all compartments although this increase was particularly marked in the case of granulocyte ferritin. It can be seen from this table that reasonable agreement was observed between whole blood ferritin calculated indirectly from measurements on isolated fractions and that determined by direct measurement.

Studies in patients with increased erythropoiesis showed appreciably higher concentrations of ferritin in the reticulocytes as compared with mature erythrocytes. In one patient, the ferritin concentration of a fraction containing 99% reticulocytes was 2.5 times the concentration in the mature red cell fraction. In a second subject, the ferritin concentration of a reticulocyte-rich fraction (56% reticulocytes) was 1.3 times the value in the adult red cell fraction. In a third subject, a red cell fraction containing 74% reticulocytes had a ferritin concentration of 4.3 times the corresponding adult red cell fraction.

The effect of inflammation on the cellular concentration of ferritin was further investigated in six patients undergoing surgery (Fig. 2). The white blood counts in these patients increased from a preoperative mean of 5,100/mm^3 to a mean of 11,300/mm^3 within 6 hr of the surgical procedure and then slowly fell to normal over the subsequent 4 days. A more gradual change in the serum ferritin level was observed in these patients, increasing from a preoperative mean of 102 ng/ml to maximum of 190 ng/ml on the third postoperative day. The pattern with mononuclear ferritin concentration was similar although the increase from a preoperative value of 45,000 ng/ml to a peak of 85,000 ng/ml occurred on the second postoperative day. The ferritin concentration in granulocytes decreased during the early postoperative period. This fall may be explained by the influx of young granulocytes containing a lower concentration of ferritin. The ferritin concentration in granulocytes subsequently rose to a maximum of 55,000 ng/ml on the second postoperative day. No consistent changes were observed in the ferritin concentration of adult erythrocytes in these patients.

Discussion. Ferritin, which represents the major form of storage iron, is found in all body cells. Roughly one-third of storage iron, or approximately 300 mg of iron in normal male adults, is in the form of ferritin.

Certain tissues, namely reticuloendothelial cells and hepatocytes, are known to account for most of this ferritin. Until recently, it has been assumed that ferritin in normal individuals was located exclusively within cells; However, with sensitive assay methods, small amounts of ferritin have been consistently detected in circulating plasma. The present study was undertaken to determine the relationship between this extravascular compartment and the formed elements of blood.

Certain limitations in the reported measurements of cellular ferritin should be noted. The mononuclear layer in this study contained both monocytes and lymphocytes and because no attempt was made to separate these two cell species, it is possible that their ferritin concentration differs significantly. The concentration of ferritin in platelets was too low to yield precise estimates, although measurements were adequate to exclude any appreciable contribution by platelets to whole blood ferritin levels. Since it was difficult to obtain reticulocytes free of granulocytes, adult red cells were used for analysis, although it was recognized that activity in the red cell fraction might be underestimated. Some check on the validity of the individual results was provided by comparison between direct measurements of whole blood ferritin and the sum of ferritin levels in the individual blood compartments (Table I). Reasonable agreement was found in the patient groups studied, with the exception of patients with iron overload.

In normal subjects the contribution of granulocytes, monocytes, serum and red cells to whole blood ferritin are roughly equal. The actual concentration of intracellular ferritin is much higher in both the granulocyte and mononuclear fraction as compared with plasma and red cells. Variations in body iron influence the ferritin concentration of nearly all cellular elements. However, the sharpest demarcation between these disorders was provided by the serum ferritin level.

With inflammation, a block occurs in the release of iron from the RE cell to transferrin. This decrease in serum iron has been shown to be associated with an increase in serum ferritin levels (7). In the present study two groups of patients with inflammation were studied, one with acute bacterial infection and the other with inflammation induced by an operative procedure. In both groups the ferritin concentration in the leukocyte fractions was moderately increased.

These studies suggest no unique participation of the formed elements in ferritin metabolism. Changes observed with variations in body iron appear to be those occurring in body tissues in general. There was some indication that monocytes may reflect, at least in part, the changes expected in the concentration of RE ferritin which derives its iron from senescent red cells. Thus, the ferritin concentration was not measurably decreased in these cells in iron deficiency, but was appreciably increased with inflammation.

Summary. The ferritin concentration of the formed elements of the blood has been measured. Nearly equal amounts were found in plasma, red cells, granulocytes, and in the mononuclear (monocyte and lymphocyte) cell fraction. Platelets contained a negligible amount. Concentrations of ferritin were greatest in blood leukocytes, amounting to approximately 24 μg/ml. Changes occurring with iron depletion and iron overload were consistent with those expected in body tissues in general. With infection, increased levels of ferritin were observed in the mononuclear cell fraction, consistent with that which occurs in RE cells.

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