SERUM FERRITIN CONCENTRATION AND ISOFERRITIN PATTERNS DETECTED BY IMMUNORADIOMETRIC ASSAY

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(Received November 14, 1979)

Summary Two-site immunoradiometric assay for serum ferritin was developed by anti-human liver ferritin antiserum applied on polyvinyl V-bottom microtiter plates. The coefficient of variation was 4.4–5.9% of liver ferritin dissolved in 1/4 diluted human serum at a concentration of 0.625–125 ng/ml. The lowest concentration (0.625 ng/ml) was statistically distinguishable from the background by Student’s t test (p<0.05). Isoferritin patterns of serum ferritin and tissue ferritins were examined by the combination of gel isoelectric focussing and 2-site immunoradiometric assay in order to explore any similarities in pIs within these ferritins. Although serum ferritin had wide range of pIs, its basic isomers corresponded well to those of liver and spleen ferritin. Geometric means and their 95% confidence limits in serum ferritin concentration were 70 ng/ml and 12–411 ng/ml, respectively, in sera of 205 healthy males (16–25 years), and 12 ng/ml and 1–211 ng/ml, respectively, in 421 healthy females (16–59 years).

Keywords serum ferritin, isoferritin, anti-human liver ferritin, immunoradiometric assay, gel isoelectric focussing, pI, liver ferritin

Ferritin is a high molecular weight iron-containing protein, which is distributed intracellularly in many tissues and can frequently be found by conventional methods in the sera of patients with certain liver diseases (1). However, recent progress in immunoradiometric assay disclosed it as a common component of all sera (2–6) and showed its parallel relation to the amount of storage iron (1, 4, 7–9). Accordingly, serum ferritin concentration has drawn attention as being a reliable indicator in evaluating iron nutrition. The present study describes a modification of 2-site immunoradiometric assay for ferritin using polyvinyl V-bottom microtiter plates (10) coated by anti-human liver ferritin antiserum as an immunoadsorbent, and discusses the rationality of the antiserum

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for assay, especially in evaluating the amount of storage iron. Serum ferritin of as many as 718 people was measured by the authors and the normal range in healthy urban Japanese of both sexes was observed in relation to Hb concentration.

EXPERIMENTAL

Preparation of human liver ferritin. Ferritin was prepared from livers obtained at autopsy of corpses with no apparent sign of liver disease. A part of the liver was homogenized with 4 volumes of distilled water, heated to 72–75°C for 10 min and centrifuged at 1,500 × g for 20 min. The supernatant was then treated twice with ammonium sulfate precipitation and ultracentrifuged by a preparative ultracentrifuge (55 p-2, Hitachi, Tokyo). The precipitate was further dissolved in 15 mM phosphate-buffered saline (pH 7.4, PBS buffer) and chromatographed successively on columns of Sepharose 6B and Sephadex G-200 (Pharmacia Fine Chem., A. B., Sweden), which were previously equilibrated using the same buffer (5, 11–14). Ferritin fraction was concentrated by an ultrafiltration cell using PM10 Diaflo membrane (Amicon, Far East, Tokyo), and the protein content was determined by the micro-Kjeldahl method. The purity of the specimens was examined by the electrophoresis on 6% polyacrylamide gel (pH 8.3) to check the correspondence of the bands, which were stained for protein by Coomassie blue and for iron by Prussian blue. Ferritins from spleen, bone marrow and kidneys were prepared principally by the same method.

Preparation of anti-human liver ferritin antiserum. One mg of purified liver ferritin dissolved in 0.5 ml of PBS buffer was mixed with the same volume of Freund’s complete adjuvant (Difco Lab., Michigan, U.S.A.). The mixture was then injected subcutaneously into two diametrically opposite sites in the backs of adult male white rabbits once weekly for 4 weeks (14). The sera obtained a week after the last injection usually had adequate activity for quantitative immunoreaction and was stored at -25°C.

Preparation of anti-human liver ferritin antibody. Human liver ferritin was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the instructions in the Pharmacia manual. After a complex had been formed between the bound ferritin and the antibody, the antibody was isolated from the immune complex by 3M sodium thiocyanate (5). It was immediately dialysed against PBS buffer and concentrated by Collodion Bag (SM 13200, Sartorius Membrane Filter, Goettingen, West Germany).

Iodination of anti-ferritin antibody. Two hundred microliters of concentrated antibody (2.2 mg/ml protein) was iodinated with 0.3 mCi of Iodine-125 (New England Nuclear, Massachusetts, U.S.A., distributed by the Japan Radioisotope Association, Tokyo) using the method of Hunter and Greenwood (15). After the reaction was stopped by sodium metabisulfite, the reaction mixture was diluted with 200 µl of 50 mM phosphate buffer, pH 7.4, which contained 0.5 mM sodium iodide and 1% bovine serum albumin. The labeled antibody was separated from
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free Iodine-125 by chromatography on a Sephadex G-100 column (Pharmacia) using BSA buffer as eluent (BSA buffer is composed of 50 mM phosphate buffer containing 4.5 g/liter sodium chloride, 1 ml/liter normal rabbit serum, 1 g/liter bovine serum albumin and 200 mg/liter sodium azide, and is adjusted to pH 7.4). The labeled antibody was further dialysed against BSA buffer and stored at -25°C (3, 5).

Separation of ferritin from human serum by immunoadsorbent. A 0.4-ml aliquot of the antiserum was coupled to 2.0 g of CNBr-activated Sepharose 4B. The bound antiserum was suspended in 200 ml of normal human sera by means of a rotating mixing device at 4°C for 24 hr. After the separation of the serum ferritin by 3 M sodium thiocyanate, the ferritin was dialysed against BSA buffer and concentrated by Collodion Bag. The ferritin-free sera that remained were used as a component of the standard medium for the assay.

Two-site immunoradiometric assay for ferritin. Polyvinyl V-bottom microtiter plates (Cooke Lab. Prod., Virginia, U.S.A.) served as the solid phase of the immunoadsorbent (10). The microtiter wells were filled with 1/5,000 diluted antiserum by PBS buffer and kept at 4°C for 24 hr. The antiserum was then removed by suction and the plates were triple washed with PBS buffer. The plates maintained activity for up to two weeks when stored in a chilled wet chamber. Ferritin standards were made by a series of 10 concentrations of human liver ferritin from 0.625 to 125 ng/ml, which were dissolved in 1/4 diluted ferritin-free serum by BSA buffer. A 200-µl aliquot of the ferritin standard or 1/4 diluted serum specimen with BSA buffer was poured into the wells and stored at 4°C for 24 hr. The specimens were then removed by suction and the wells were triple washed with PBS buffer. A 200-µl sample of 125I-labeled antibody (50,000–80,000 cpm) was placed in the wells and again kept for 24 hr. After the wells had been washed thoroughly with PBS buffer, the radioactivity in each well was measured by Aloka Autowell Gamma System (ARC 221, Aloka, Tokyo) and the results were expressed as the mean of four to six replicated specimens.

Gel isoelectric focussing and isoferritin detection. Isoelectric focussing of ferritin was performed in 4% polyacrylamide gel containing 3% ampholine composed of a 1:1 mixture of pH 4–6 and 5–7 (LKB Produkter, Bromma, Sweden) at 4°C (16, 17). After 10 min, prerunning, 50 µl of the specimen (100–250 ng ferritin) was placed on the cathodic end and a current of 1 mA/gel was applied until the voltage reached 400 V, then the voltage was kept steady for 7 hr. The gels were then sliced into 1.0 mm slabs by a YH slicer (Hotta Rika, Tokyo), and the ferritin in individual slices was assayed by 2-site immunoradiometric assay after the slices had been inserted into separate wells containing 200 µl of PBS buffer. The pH of the gels was measured in another set of the slices using a research pH meter (PHM 64, Radiometer, Denmark).
RESULTS

Standard curve and accuracy

A standard curve was made from a series of 10 concentrations of human liver ferritin from 0.625 to 125 ng/ml dissolved in 1/4 diluted ferritin-free human serum (Fig. 1). Each point represented the mean of 6 replications, and standard deviation of the mean is shown by a bar at each point. The radioactivities had a positive relation to ferritin concentrations and the lowest concentration (0.625 ng/ml) was significant different from the background ($p<0.05$) by Student's t test. Coefficient of variation ($SD \times 100/mean$) showed a marked narrow range of 4.4–5.9%. Accordingly, this method could measure serum ferritin reliably at these concentrations, which might practically cover the range which appears in normal or iron-deficient subjects.

Patterns of isoforms from serum and tissues

Patterns of isoforms from serum and tissues such as liver, spleen, bone marrow and kidneys were determined by the method described in this paper. Each pI was defined by the peak which projected above its surroundings in more than two sequential determinations. Liver ferritin demonstrated 5 distinct peaks of pI 5.78, 5.71, 5.62*, 5.53* and 5.46 (asterisks indicate main peaks) (Fig. 2). Serum ferritin had a wide range of 6 pIs consisting of 5.78*, 5.64*, 5.53, 5.39, 5.12 and 4.85 (Fig. 3). The pIs of various ferritins are summarized in Table 1. While

![Fig. 1. Standard curve of 2-site immunoradiometric assay for human liver ferritin is shown as a plot of radioactivity versus log dose. Ferritin is dissolved in 1/4 diluted ferritin-free human serum with BSA buffer. Standard deviations are shown for six replications of each point.](image)
Fig. 2. Isoferritin pattern of normal human liver ferritin is shown by the combination of gel isoelectric focussing and 2-site immunoradiometric assay (see text). The pI values of isoferritin are indicated in the figure. — Indicates Iodine-125 activity at CPM and ○○ indicates pH value.

Fig. 3. Isoferritin pattern of normal human serum. The method and symbols used in this experiment are the same as those in Fig. 2.

Isoferritins from liver were more basic than other ferritins, serum ferritin showed the most acidic isomers of pI 5.12 and 4.85 of all specimens. It is noteworthy that anti-liver ferritin antiserum has the capacity to interact with acidic isomers which are generally not recognized in liver ferritin. Furthermore, serum ferritin corresponded to liver ferritin at 3 pIs containing a main peak, and also to spleen.
Table 1. pI values of isoferritins derived from normal human serum and organs.

| Serum  | Liver | Spleen | Bone marrow | Kidney |
|--------|-------|--------|-------------|--------|
| 5.78*  | 5.78  | 5.78   | 5.78*       |        |
| 5.71   |       | 5.70   |             |        |
| 5.64*  | 5.62* | 5.62   | 5.62*       | 5.63*  |
| 5.53   | 5.53* | 5.52*  | 5.52*       | 5.53*  |
| 5.39   | 5.38* | 5.44   | 5.47        | 5.27   |
| 5.12   |       | 5.19   | 5.20        |        |
| 4.85   |       |        |             |        |

* Major isoferritin peaks.

ferritin at 4 pIs. These results suggest the rationality of the use of anti-liver or anti-spleen ferritin antiserum in serum ferritin assay, especially in cases of storage iron evaluation.

Serum ferritin concentration of both sexes

The distribution of serum ferritin in apparently healthy nonanemic subjects whose hemoglobin concentration was above 13 g/100 ml in males and 12 g/100 ml in females is shown separately in the 205 healthy males (16–25 years old) and 421 females (16–59 years old). Serum ferritin distribution was skewed in both cases (Figs. 4 and 5). Geometric means and 95% confidence limits were 70 ng/ml and 12–411 ng/ml in males, respectively, and 12 ng/ml and 1–211 ng/ml in females, respectively (Table 2). The mean concentration of the males was significantly higher than that of the females (p<0.001). The median value was 68 ng/ml in males and 16 ng/ml in females.

The relation between serum ferritin concentration and hemoglobin value

The relation between these two conditions was determined by the quadrants made by the lines drawn horizontally at serum ferritin 12 ng/ml and vertically at hemoglobin value 13 g/100 ml in males or 12 g/100 ml in females (Figs. 6 and 7). First, IIInd, IIIrd and IVth quadrants were supposed to indicate normal iron nutrition, latent iron deficiency, iron-deficient anemia and anemia other than iron deficiency, respectively. The results obtained by the survey are summarized in Table 3. A marked difference between the sexes was the tendency in females of latent iron deficiency. Whereas 94% of the males had normal iron nutrition, 41% of the females suffered from latent iron deficiency and their normal iron nutrition decreased to about 48%.

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DISCUSSION

Since sensitive methods for serum ferritin assay were developed (2–6), ferritin has proven to be a normal constituent of serum and its concentration has been found in the range of 12–300 ng/ml in normal subjects (4). Furthermore, the presence of a positive relation between serum ferritin concentration and amount of storage iron was recognized by various parameters which were adaptable to the estimation of storage iron, that is, repeated phlebotomy, iron measurement in liver biopsy tissue or bone marrow aspirate, and rate of iron absorption (1, 7–9). Owing
Table 2. Geometric mean, 95% confidence limits and median of serum ferritin is shown in apparently healthy nonanemic males and females.

|       | No.  | Age   | Geometric mean | 95% Confidence range | Median |
|-------|------|-------|----------------|-----------------------|--------|
| Male  | 205  | 16–25 | 70 ng/ml       | 12–411 ng/ml          | 68 ng/ml |
| Female| 421  | 16–59 | 12 ng/ml       | 1–211 ng/ml           | 16 ng/ml |

Fig. 6. The relation between serum ferritin concentration and hemoglobin value in 93 high school boys. Four quadrants are made by crossing the lines of serum ferritin at 12 ng/ml and hemoglobin value at 13 g/100 ml.

Fig. 7. The relation between serum ferritin concentration and hemoglobin value in 240 high school girls. Four quadrants are made by crossing the lines of serum ferritin at 12 ng/ml and hemoglobin value at 12 g/100 ml.
Table 3. The relation between serum ferritin concentration and hemoglobin value.

|                | No. | I   | II  | III | IV  |
|----------------|-----|-----|-----|-----|-----|
| Female         |     | I   | II  | III | IV  |
| High school girls | 240 | 52.3% | 40.7% | 3.3% | 3.7% |
| Nurse school students | 115 | 29.6% | 50.4% | 13.9% | 6.1% |
| Well-trained volleyball players | 88 | 58.0% | 28.4% | 6.8% | 6.8% |
| Middle-aged    | 31  | 54.8% | 41.9% | 3.2% | 0.0% |
| Total female   | 474 | 47.9% | 40.9% | 6.5% | 4.6% |
| Male           |     |     |     |     |     |
| High school boys | 93 | 94.5% | 2.2% | 2.2% | 1.1% |
| Students and staffs in medical school | 117 | 94.0% | 4.2% | 0.9% | 0.9% |
| Total male     | 210 | 94.3% | 3.3% | 1.4% | 1.0% |

Four quadrants are made by crossing the lines of serum ferritin at 12 ng/ml and hemoglobin value at 13 g/100 ml in males or 12 g/100 ml in females. I, nonanemic; II, latent iron deficiency; III, iron-deficient anemia; IV, anemia other than iron deficiency.

to its technical readiness and high reproducibility, serum ferritin assay has been widely used by many investigators as a reliable method for evaluation of storage iron of subjects having various iron states. Meanwhile, the abnormal increase of serum ferritin has been observed in patients with liver diseases or malignant tumors (1, 18–20), which was thought not to relate directly to body iron status. The latter cases may suggest derangement of ferritin transport through cell membranes or heterogenous synthesis of some specific isoferitins in certain tissues from which a large part of the serum ferritin had originated. Such abnormal increases might be considered to be a problem differing from normal iron metabolism. The methods employed for serum ferritin assay are principally divided into the following categories: immunoradiometric assay, including the 2-site method (2–6); double antibody radioimmunoassay (18); and enzyme immunoassay (19). The authors employed the 2-site immunoradiometric assay, which was modified by the use of polyvinyl V-bottom microtiter plates instead of polystyrene or polyethylene tubes as the solid phase of immunoadsorbent (10). The advantages of the modification were ability to simultaneously measure large numbers of specimens, and economy compared to the use of full-size plastic tubes. The sensitivity and accuracy required for the assay was fully satisfied by this method.

Heterogeneities of immunoreaction between different types of ferritins and their antisera have already been mentioned by the investigators (1, 21–23). Ferritins originating from liver or spleen are composed of basic isoferitins, while those from heart or HeLa cells contain acidic isomers. The complex formed between the same
type of antigen and antibody was more plentiful than that formed between their alternatives. Originally, the antiserum for serum ferritin assay should be made by immunization with the same antigen, but the yield of serum ferritin in the purification procedure is too minute for the purpose. Accordingly, serious consideration was required in the choice of an antiserum suitable for this purpose. The authors examined patterns of isoferritins derived from several tissues and combined sera of healthy males with the combination of gel isoelectric focussing and 2-site immunoradiometric assay to compare pls from each ferritin (Table 1). Although serum ferritin was prepared by immunoadsorption for anti-liver ferritin antiserum, the pattern indicated the presence of a wide range of pls including not only the basic isomers which corresponded to those from liver or spleen but also the most acidic isomer tested. There was a high coincidence in pls between the basic isomers of serum ferritin and liver or spleen ferritin. These results are believed to suggest the adequacy of anti-liver or anti-spleen ferritin antiserum for serum ferritin assay, especially in the case of storage iron evaluation.

The results obtained by the authors were derived from urban Japanese in Tokyo and its outskirts. When the values are compared to those of other investigators, the average value of the males 70 ng/ml* was indistinguishable from those of others, 52, 56.9*, 85.1*, 94* and 123 ng/ml. The asterisks indicate the geometric means. In contrast, the average value of the females, 12 ng/ml* seemed to be lower than those of others 28.8, 34.0*, 34*, 39.8* and 56 ng/ml (2, 5, 6, 8, 24). These results are believed to show an iron status in urban Japanese which is particularly inadequate iron nutrition in the females of this area.

We would like to thank Dr. Y. Kuroda (Chairman, sports-science committee of Japan amateur sport association) and Dr. S. Nagamine (Head of research project for the prevention of sport anemia) for their kind cooperation in receiving specimens from volleyball players.

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