A Screening Test for Assessing Iron Status

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Iron deficiency is highly prevalent in many developing countries, affecting from 10% to 30% of the population in some areas. In order to alleviate this nutritional deficiency, additional iron must be supplied to the population either with the diet (iron fortification) or as iron medication (iron supplementation). However, a significant concern with these intervention strategies is that certain individuals may be adversely affected by a higher iron intake. For example, in Thailand where iron deficiency is very common, about 1% of the population have inherited abnormalities in hemoglobin synthesis (e.g., thalassemia major, hemoglobin E-thalassemia) that may lead to early death from iron overload. Many of these disorders are associated with a hypochromic microcytic anemia, making it difficult to distinguish them morphologically from iron-deficiency anemia.

Serum ferritin levels provide a clear separation between iron-deficiency anemia (below 20 μg/liter) and thalassemia major (greater than 500 μg/liter). Most conventional radioimmunometric assays for serum ferritin require several hours to perform and are not practical from iron-deficiency anemia. The procedure, which can readily distinguish iron deficiency from even a modest increase in storage iron, has a potentially wide application in settings where a prompt assessment of iron status is required.

Materials and Methods

Human Ferritin

This was purified from human liver tissue using a modification of the method of Mazur and Shorr. Following six recrystallizations with cadmium sulfate, the material was passed through a Sephadex G200 column and concentrated by ultracentrifugation at 100,000 g for 2 hr. The ferritin button was dissolved in normal saline containing 0.2% sodium azide and stored at 4°C. The concentration of ferritin protein was determined by the Lowry method using bovine serum albumin (BSA) as the standard.

Rabbit Antiferritin

Rabbits were immunized by injecting 200 μg ferritin with complete Freund’s adjuvant at weekly intervals for 3 wk followed by one final injection 3 wk later. The titer of whole antisera used in these studies was 1:32. Crude antisera were stored at -20°C.

Antiferritin Antibody

This was isolated by affinity chromatography using cyanogen bromide-activated Sepharose 4B as described by Gonyea. The protein yield was assessed by determining absorbance at 280 nm. Ten milliliters whole antisera yielded approximately 15 mg protein, which was then diluted to a final concentration of 10 mg/ml.

Enzyme-Conjugated Antibody

Purified antiferritin was conjugated with alkaline phosphatase using a modification of the method of Theriault and Page. Ten milligrams purified antiferritin was added to 0.6 ml alkaline phosphatase in 3.2 M ammonium sulfate (alkaline phosphatase from calf mucosa, 5050 U/ml; Sigma Chemical Co., St. Louis, Mo.). This mixture was dialyzed against 1 liter phosphate-buffered saline (PBS) (15 mmole/liter pH 7.2, containing 0.2% sodium azide). The antibody-enzyme mixture was then mixed with 100 μl glutaraldehyde solution (10 g/liter water) and allowed to stand 2 hr at room temperature. After further dialysis against PBS, the material was applied to a Sephadex G200 column that had been previously equilibrated with PBS. Eluted fractions with the highest protein content as measured by absorbance at 280 nm were pooled; the conjugate was diluted 1:10 with 5% BSA in 0.05 M Tris-HCl, pH 8.0, and stored at 4°C.

Preparation of Solid Phase

The test was performed using polystyrene microtiter plates (Dynatech Laboratories, Alexandria, Va.). The solid phase was prepared by adding to each well whole rabbit antiferritin serum diluted 1:1250 with 0.2 M sodium bicarbonate, pH 9.2. After standing overnight at 4°C, the serum was aspirated and the wells washed 3 times with PBS. The washed plates were covered with sealing film (Dynatech Laboratories, Alexandria, Va.) and were stored in this manner for up to 12 wk at 4°C.

Assay Procedure

In the first step, two drops containing approximately 35 μl serum are added with a Pasteur pipette to each well followed by the same volume of BSA-buffer (30 g BSA/liter PBS). Standards are similarly prepared by adding 2 drops ferritin solution to each well followed by 2 drops normal rabbit serum. After allowing the plates...
to stand at room temperature for 15 min, the wells are aspirated and rinsed 3 times with PBS.

In the second step of the assay, 4 drops conjugated antiferritin (diluted 1:100 in BSA buffer) are placed in each well and the plates allowed to stand for 15 min. The wells are again aspirated and rinsed 3 times with PBS.

In the final step, each well is filled with substrate solution containing p-nitrophenylphosphate (PNP) prepared by dissolving 2.0 g PNP in 100 ml 1 M diethanolamine containing 0.1 g/liter magnesium carbonate, pH 10.0. After allowing the plates to stand at room temperature for 1 hr, the reaction is stopped by adding one drop of 1 M sodium hydroxide to each well. The intensity of the yellow color in each well is estimated visually against a white background. When the test is performed with whole blood rather than serum, capillary blood is drawn into heparinized microhematocrit tubes (Fisher Scientific, St. Louis, Mo.). The contents of one capillary tube are placed in each well followed by 2 drops of BSA-buffer. For standards, 1 drop ferritin solution is added to each well together with 1 drop buffer and 2 drops heparinized whole rabbit blood. The plates are incubated, washed, and aspirated as described above.

RESULTS

In the initial evaluation of the assay, serum from venous blood was used for both the screening test and quantitative measurements of serum ferritin using a conventional immunoradiometric assay (IRMA). The color intensity in the screening test with each sample was compared to ferritin standards containing 0, 20, 50, 100, 200, and 500 μg/liter. Color intensity of each well was assessed independently by 5 individuals. The results of a study on 90 sera are shown in Fig. 1. While some overlap was observed in the screening test, the feasibility of the approach was established.

In a second evaluation, attention was focused on the screening assay as a means of distinguishing iron deficiency from iron overload in patients with anemia. To further simplify the procedure, the need for centrifugation was eliminated by using heparinized whole blood obtained by fingerstick. The results with the screening test were compared to serum ferritin levels measured with IRMA on venous blood drawn simultaneously. On the basis of ferritin standards containing 20 and 200 μg/liter, the color was judged to be in one of three categories: low (<20), normal (21–200), or elevated (>200); color was estimated by 5 observers on samples from 38 patients with a wide range in serum ferritin.

Using the broader ranges of low, normal, and high,
excellent agreement was obtained between the screening test and the IRMA method (Fig. 2). Of 4 samples with low serum ferritin (8, 10, 13, and 20 µg/liter), 90% were correctly placed by the screening test, and in no instance was the ferritin estimated as high (Table 1). Of 12 sera with ferritins between 21 and 200 µg/liter, 92% were correctly assigned. With 22 sera containing over 200 µg/liter serum ferritin, 98% were identified correctly and the ferritin was not judged low in any of these.

**DISCUSSION**

The prime objective of this study was to develop a simplified test that could readily distinguish iron-deficiency anemia from anemia associated with iron overload. This objective was achieved; in no case was a low ferritin considered high or vice versa (Table 1). It should be noted that not all patients with iron-deficiency anemia have ferritin values below 20 µg/liter. When iron lack is associated with inflammation, infection, or liver disease, serum ferritin levels as high as 60 µg/liter may occur in anemic patients with absent iron stores. However, because ferritin levels in patients with significant iron overload are invariably above 500 µg/liter, the screening test can still distinguish iron deficiency from iron excess.

An important advantage of the screening test is that it can be performed without laboratory facilities and is therefore suitable for use in field work and in rural health clinics. Although sophisticated laboratory facilities are required to prepare the materials for the assays, the coated plates and reagents can be stored for at least 12 wk without appreciable deterioration. From a practical aspect, the screening test involves a total incubation time of 90 min, although one person can easily perform 15–20 tests/hr. This incubation time could be shortened appreciably with certain modifications in the procedure. For example, we have found...
that incubation at 37°C rather than room temperature allows a decrease in the incubation time with substrate from 60 to 30 min. We did not employ the higher temperature since this would be inconvenient in field work. An alternate approach would be to employ double monoclonal antibodies against ferritin, one to prepare the solid phase and one for enzyme conjugation. This would allow simultaneous addition of the unknown and the enzyme-conjugated antibody, and thereby eliminate one phase of the test. The paradoxical fall in the dose–response curve seen with IRMA when large doses of standard are used is not seen with the screening test, and color development is not appreciably altered when whole blood is used because the red cells are removed after the first incubation step. However, it is necessary to ensure that the volume of standard solution used is approximately equivalent to the serum content of whole blood.

Although the screening test was designed to distinguish between the extremes in iron status in a setting with limited laboratory facilities, it has several potential applications in the United States. Since all causes of anemia except for iron deficiency are associated with an increase in serum ferritin, the absence of color in the screening test would provide strong presumptive evidence of iron deficiency. By performing the test in all patients admitted to hospital with low hemoglobin levels, a more costly hematologic evaluation could be avoided. Another application would be to detect latent iron deficiency in nonanemic patients in segments of the population where iron deficiency is uncommon. If performed in conjunction with stool tests for occult blood as part of the annual medical examination, the early recognition of a gastrointestinal malignancy would be facilitated.

A screening test for iron status might also be useful in a community blood bank where potential donors are presently screened by hemoglobin or hematocrit determinations, which are relatively insensitive measures of iron deficiency. The test would facilitate the identification of nonanemic donors with latent iron deficiency who may develop frank anemia with continued blood donations. Another useful application for this test is in those segments of the population susceptible to iron deficiency, such as women of childbearing age. Since many women are reluctant to take iron supplements during pregnancy because of associated gastrointestinal side effects, the screen could be used in prenatal clinics to identify those women with a specific need for iron therapy.

REFERENCES

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