A Solid-Phase Enzyme Immunoassay for Serum Ferritin

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We describe an enzyme immunoassay for human serum ferritin in which antibody adsorbed on polystyrene tubes is used. Adsorbed gamma-globulins against human ferritin were first allowed to react with ferritin and a second antiferritin antibody, labeled with alkaline phosphatase, was added. The amount of bound enzyme/antibody conjugate was proportional to the ferritin titer in the assay. This method offers stable reagents that can be kept for many months at 4 °C. The average values for ferritin in normal men and women were, respectively, 58 and 43 µg/liter. The lowest detectable concentration was 5 µg/liter.

Ferritin, in its various forms, plays a very important role in iron storage and metabolism (1). It is present in various tissues but especially in liver, spleen, and bone marrow (1). The presence of ferritin in certain tumors and the increased titer of this protein in the serum of cancer patients led some investigators to postulate that ferritin could be used as a marker for cancer (2–4). Radioimmunological assays for ferritin have been reported (4–8) but the frequent radiolabeling of the material, which is not always suitable for the routine clinical laboratory, led us to develop the enzyme immunoassay described below, which involves stable reagents.

Materials and Methods

**Human ferritin.** Ferritin was isolated and purified from normal human liver by a modified method of Penders et al. (9) as follows. Liver was homogenized in two volumes of distilled water and the homogenate was heated at 75 °C for 15 min. The ferritin was separated from the coagulated proteins by filtration on a Büchner filter and the solution was concentrated by ultrafiltration through an Amicon PM-10 membrane and ferritin then prepared by ultracentrifugation as described by Penders et al. (9). The last residue was dissolved in the smallest possible volume of isotonic sodium chloride and chromatographed on Sephadex G-200 (Pharmacia Canada Ltd.); the ferritin, which appeared in the void volume, was then chromatographed on Sepharose 6B (Pharmacia Canada Ltd.) in isotonic sodium chloride.

**Anti-human ferritin.** Each rabbit was first injected subcutaneously on both sides with 0.2 ml of a 1:1 mixture of complete Freund's adjuvant and a purified human ferritin solution, 2 g/liter, as determined by a modified Lowry method (10). On the second and third week, the rabbits were intramuscularly administered the same amount of ferritin without adjuvant. Animals were bled out on the fifth week and the antisera was stored frozen.

**Antibody/enzyme conjugate.** Gamma globulins were prepared from antiferritin serum by salt precipitation in one-third saturated ammonium sulfate; the procedure was repeated three times and the residue was dissolved in water and dialyzed for 24 h against three changes of 10 liters of phosphate-buffered saline (15 mmol/liter, pH 7.2, and containing sodium azide, 1 g/liter) (11). Six hundred microliters of alkaline phosphatase in 3.2 mol/liter (NH₄)₂SO₄ (from calf mucosa, 5 g/liter, 520 U/mg; Sigma Chemical Co., St. Louis, Mo. 63178) was added to 1 ml of the dialyzed gamma-globulins (10 g/liter). Ammonium sulfate was eliminated from the final solution by dialysis against 1 liter of the above buffer, and 100 µl of a glutaraldehyde solution in water (10 g/liter) was then added, as described by Engvall et al. (12). The solution was left for 2 h at room temperature and the conjugate was separated from free glutaraldehyde on a Sephadex G-50 column (Pharmacia Canada Ltd., 0.9 x 15 cm) in phosphate-buffered saline. The portion of the eluate corresponding to the excluded volume of the column was taken and used as the antibody/enzyme conjugate.

The conjugate was then analyzed by Ouchterlony double diffusion against pure ferritin; for staining we used a histochemical technique with Naphthol AS-MX substrate coupled to Fast Blue RR salt (Sigma) (13).

**Solid-phase assay.** Polystyrene test tubes (Falcon, Oxnard, Calif. 93030; No. 2052, 12 x 75 mm) were coated with 1 ml of 1100-fold dilution of the antiferritin gamma-globulins (12 g/liter) in phosphate-buffered saline (pH 7.2, containing sodium azide, 1 g/liter). The tubes were left overnight at 4 °C; then the solution was decanted and the tubes were washed five times with 2-ml portions of phosphate-buffered saline at room temperature.

Either 1 ml of standard ferritin solutions in phosphate-buffered saline containing bovine serum albumin (40 g/liter; Sigma; Cohn Fraction V) or 1 ml of different dilutions of serum was added to the coated tubes, in duplicate. After an overnight incubation at room temperature, the solution was decanted and the tubes were washed five times with 2-ml portions of phosphate-buffered saline. One milliliter of a 250-fold dilution of the enzyme/antibody conjugate solution (2 g/liter) in phosphate-buffered saline containing bovine serum albumin was then added and the tubes were incubated overnight at room temperature, then decanted and washed as described previously.

The enzyme activity was measured (15) by adding 1 ml of p-nitrophenyl phosphate (1 g/liter) in 2-amino-2 methyl-1-propanol buffer (625 mmol/liter, pH 10.25; Technicon Instruments Corp., Tarrytown, N.Y. 10591). After an hour of
incubation at 37 °C in a shaking water bath, the reaction was stopped by adding 100 μl of 4 mol/liter NaOH and the absorbance was measured at 410 nm with a Beckman DBGT spectrophotometer.

Results

Antiferritin serum. Figure 1 shows the specificity of the antiserum obtained in a rabbit inoculated with the human ferritin isolated as described above. A faint precipitation line is obtained after migration against commercial horse ferritin (Sigma).

Solid-phase assay. Figure 2 shows the standard curve obtained under the described conditions. The reaction gives a plateau for values exceeding 100 μg/liter. Intra-assay variation was less than 11% for high and low ferritin values. The value of each duplicate is shown on the figure; the standard curve is the best fitting line between the mean of the duplicates. The difference in absorbance between standard solutions containing, respectively, 0 and 5 μg/liter of ferritin still exceeded 0.025 A. When the antiserum was in the tubes at -20 °C for as long as 36 days, results could be reproduced with no substantial loss of immunological activity or sensitivity. As shown in Figure 3, the curve obtained after 13 days of coating is very similar to the one obtained after a coating period of only one day. Standard curves obtained after coating periods varying from one to 36 days gave a family of curves that were all equally useful for the assay.

The dilutions of both coating antibody and enzyme antibody conjugate in this assay were chosen because of the linearity of the standard curve in the portion that corresponds to serum ferritin titers of normal individuals (Figure 4). A skewed distribution of ferritin titers in both normal men and women was observed, as has been reported by other investigators (6, 14). The geometric mean for 44 normal men was 58 μg/liter, with an acceptable range of 19 to 170 μg/liter. The geometric mean for 80 normal women was 43 μg/liter, with an acceptable range of 15 to 120 μg/liter (at the 97.5% confidence level). These data are comparable to the normal values already reported by other authors (5, 6).

Discussion

The solid-phase enzyme immunoassay described above is useful for routine use in the clinical laboratory. It eliminates the need for constant labeling of antigen or antibody with iodine-125. It is reproducible, the intra-assay variation being less than 11%. The antibody dilution was adjusted so that most sera may be assayed without dilution. The sensitivity of the method described above is inferior to some of the immunoradiometric assays reported (4-9), but it is sufficient for routine assays. All manipulations are performed within a single tube, thus avoiding errors due to losses. Tubes may be
coated in large batches and kept in the freezer for at least four weeks without significant loss of activity. The mean serum ferritin values that we obtained with this enzyme immunoassay are comparable to the ones reported by Addison et al. (5) and Halliday et al. (6), who used radioimmunochemical techniques. A comparison of the enzyme immunoassay with the radioimmunoassay has already been made for other proteins (16, 17), and our results are in agreement with the already-reported radioimmunoassay for ferritin, both for its sensitivity and reproducibility and for normal values. However, the use of enzyme for the amplification of quantitative immunoassays of proteins has the decided advantages of greater laboratory safety and economy as compared to the use of radioisotopes.

This work was supported by the National Cancer Institute and the Conseil de la Recherche en Santé du Québec.

References