Interaction Between Rabbit Erythroblast Ferritin and Normal Plasma Proteins

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Erythroblast ferritin (EF), isolated from phenylhydrazine-anemic rabbit marrow, interacts with components of normal plasma and forms a complex separable by starch granule electrophoresis and by dialysis techniques. The binding is facilitated at low ionic strength. The plasma factors responsible for binding EF are present in autologous, as well as homologous, plasma of normal nonimmune rabbits, although binding activities are quite variable in different plasmas. The binding activity for rabbit EF is also present in heterologous plasma of mouse, guinea pig, and man. The active principles in plasma were identified as two heat-stable components which fractionate as immunoglobulins, one as IgG and the other as IgM. The results support the hypothesis that natural antiferritin antibodies of low titer are present in normal plasma.

Although organ specificity of ferritin was demonstrated in 1967,1,2 little progress has since been made in elucidating the unique structural properties of the ferritin in erythroid cells. Erythroblast ferritin (EF) forms an iron pool which is physicochemically separable and metabolically distinct from that of the reticuloendothelial cells.3 Because of the rapid turnover of its iron, high-specific-activity iron labeling of EF can be readily achieved in vitro.4 Although it appears likely that EF chiefly subserves the iron requirements of heme synthesis, there is little direct experimental evidence about its role in erythropoiesis. Deiss and Cartwright have found in vitro evidence for a catabolic pathway for EF from reticulocyte to plasma to monocyte.5 The present experiments describe plasma factors capable of binding relatively small quantities of EF.

MATERIALS AND METHODS

Preparation of 59Fe-labeled EF

Marrow suspensions rich in erythroid cells were prepared from phenylhydrazine-anemic New Zealand white rabbits and the EF isolated after in vitro labeling with 59Fe. The washed cell suspensions were resuspended in Eagle’s minimal essential medium (MEM) containing normal rabbit serum labeled with 59FeCl3 (International Chemical and Nuclear Corp.; specific activity, 10-30 μCi/μg) 10 μC: per ml serum. Final mixtures contained 3 volumes cells: 2 volumes MEM: 1 volume labeled serum. Four-hour incubations with mixing at 37°C resulted in high-
specific-activity labeling of the EF. After completion of the incubation, the cells were washed, lysed with 0.01 M Tris HCl buffer at pH 7.4, homogenized and ultrasonicated, and then centrifuged at 3000 rpm for 1 hr at 4°C. The supernate was heated at 80°C for 15 min and the precipitate removed by centrifugation. The supernate was fractionated at 50% ammonium sulfate, the precipitate harvested and purified first on columns of Sepharose 6B (Pharmacia), followed by ion-exchange chromatography of the ferritin fraction on columns of DEAE Sephadex A-50. The method yields ferritin with a high degree of purity. Added details of the isolation procedure were previously described. The isolated 59Fe-EF used in these studies had an iron:protein ratio of 0.116 ± 0.016, with iron and protein content of 5.27 ± 1.4 µg/ml and 45.1 ± 9.4 µg/ml, respectively. Its specific activity was 26.1 ± 7.5 x 106 cpm per mg Fe. Some preparations were obtained from pooled marrow cells from two or three rabbits.

Test Plasma

Most studies were done on heparinized plasma from autologous and homologous rabbits, but plasma from mouse, guinea pig (obtained from Grand Island Biological Company), man, and rat were also studied. Storage of plasma was at -20°C. Rabbit plasma with high EF binding activity was chemically treated by various means for assessment of alterations of its activity. Equal volumes of 0.1 M hydrazine and plasma were incubated for 1 hr at 37°C, followed by neutralization with propionaldehyde and then by dialysis for 24 hr at 4°C against barbital buffer containing 0.15 M NaCl. Two milliliters of plasma were treated with 0.5 ml NH4OH for 1 hr at 37°C, followed by neutralization with 0.1 M HCl. EDTA, sodium cyanide, and sodium azide were added to plasma at 10⁻² and 10⁻³ M.

Active plasma was adsorbed by two different techniques. Zymosan was washed initially three times in saline before use and then added to plasma at a concentration of 100 mg/ml. Two successive absorptions were performed at 4°C. Active plasma was also adsorbed on monolayers of spleen macrophages. The spleen macrophage monolayer was formed according to the method of Abramson et al. Rabbit spleen cell suspension (0.4 ml) was added to a vinyl cup (3.5 cm in diameter). After addition of 0.1 ml Hanks' solution and 45 min standing at 24°C, the attached monolayer was rinsed with four washes of Hanks' solution. Test plasma (0.5 ml) was placed over the monolayer, incubated for 2 hr at 37°C, and the plasma removed and centrifuged. Binding activity for 59Fe-EF before and after absorption was measured by the dialysis technique.

Fractionation procedures, described below, were used to characterize the plasma components active in binding EF. Three milliliters of plasma were separated by starch granule electrophoresis and by Sephadex G-200 gel filtration, and 4 ml by DEAE-cellulose column chromatography. Separated fractions were individually assayed for EF binding activity by the dialysis technique, using mixtures of 5 µl 59Fe-EF added to the fraction volume of 1.3-2.0 ml.

Dialysis Techniques

Dialysis in low-ionic-strength buffer was used to demonstrate complex formation between EF and plasma components by virtue of the insoluble character of the complex under these conditions. After 3 hr incubation of the EF-plasma mixture, 2-3 ml of dialysis buffer were added and the contents placed in 1-inch dialysis tubing. Dialysis was continued for 24 hr against 2 liters of 0.01 M Tris HCl buffer at pH 7.4 with internal stirring. After dialysis the precipitate was separated by centrifugation at 3000 rpm for 30 min at 4°C and the supernate and precipitate separately analyzed for 59Fe. Results were expressed in terms of the per cent of the total 59Fe precipitated. Plasma was similarly separated into a globulin precipitate and a soluble supernate after dialysis, and then the separated supernate and precipitate (the latter after being redissolved in the dialysis buffer containing 0.15 M NaCl) were separately assayed for EF binding activity.

Fractionation Procedures

Starch granule electrophoresis was done at 10 V/cm for 17 hr at 4°C in 0.05 M barbital buffer at pH 8.6. After completion of the run, 0.5-cm segments were cut and the protein eluted with 2.0 ml barbital buffer containing 0.15 M NaCl. Gel filtration was performed on Sephadex G-200 in a 2.5 x 44-cm column in 0.01 M Tris HCl buffer, pH 7.4, containing 0.15 M NaCl at a con-
trolled flow rate of 0.1 ml/min. DEAE cellulose chromatography (Whatman DE 11) was performed on a 2.5 x 20-cm column. Equilibration was achieved at 0.02 M, pH 8.0, phosphate buffer, and elution was at constant pH with a linear gradient from 0.05 to 0.3 M phosphate buffer. Flow rate was 0.4 ml/min.

Miscellaneous Determinations

Protein concentration was measured by optical density at 280 m\(\mu\) or by the Lowry modification of the Folin technique.\(^7\) Radioactivity was determined in a Nuclear Chicago well scintillation counter with efficiency of 8%.

RESULTS

Complex Formation Between \(^{59}\)Fe-labeled EF and Normal Plasma Components

When purified \(^{59}\)Fe-labeled EF is added to normal rabbit plasma and the mixture separated by starch granule electrophoresis, a fraction of the EF localizes in the \(\gamma\)-globulin zone, in contrast to the EF alone, which forms a single homogeneous peak in the \(\alpha\)-globulin zone. As the ratio of plasma to EF is increased, the proportion of EF which migrates in the \(\gamma\)-globulin zone becomes greater (Fig. 1). The results resemble data previously reported with specific immune sera, except that much lower proportions of immune sera were required to achieve complete change in EF electrophoretic migration.

The interaction between \(^{59}\)Fe-labeled EF and plasma components is also shown by dialysis techniques (Fig. 2). At low ionic strength, the complex precipitates in the insoluble fraction, permitting its convenient detection. EF alone
without added plasma remains in the soluble phase. As the proportion of plasma to EF is increased, the proportion of precipitated EF increases and approaches 100%.

$^{59}$Fe-EF prepared with omission of the step requiring heating to 80°C did not affect its binding affinity for plasma components, excluding heat denaturation as a possible explanation for complex formation. Subsequent heating of the isolated EF preparation also did not influence its binding affinity.

Plasma samples from 27 nonimmune rabbits were tested for EF binding activity on starch granule electrophoresis, and all were found to possess significant activity, with a great deal of variability among the various samples. Of the 27 plasmas tested, 15 possessed only weak activity (binding only 2%–9% of the EF), six were moderately active (binding 13%–24% of the EF), while the six most reactive bound from 39% to 80% of the added EF under the experimental conditions used. The more active plasmas were used in the experiments described below. When the plasmas from the same rabbits were drawn and tested repeatedly at intervals, the binding activity of individual plasmas remained relatively constant. Complex formation was demonstrable in autologous, as well as homologous, plasmas of nonimmune rabbits. The complex formation was not species specific, since it was also demonstrated both by starch granule electrophoresis and by the dialysis technique with plasma of the mouse, guinea pig, and the human, although not of the rat.

The binding of $^{59}$Fe-labeled EF with the plasma component is facilitated by dialysis of the mixture in low-ionic-strength buffer. As the concentration of NaCl is increased, the degree of complex formation decreases (Fig. 3). EF in the absence of plasma did not significantly precipitate at any ionic strength. The effect of pH on the complex formation during dialysis of EF-plasma mixtures is demonstrated in Fig. 4. The most marked precipitation of EF occurred at pH 5.0, but it also was observed at this pH in the absence of plasma and thus did not resemble the complex described, which precipitated at an optimum pH of 8.0. Precipitation of EF in the absence of plasma was negligible above pH 6.5.

The complex was analyzed to ensure that the $^{59}$Fe tag had indeed remained associated with EF during the experiments. The complex was first separated by starch granule electrophoresis. The eluted peaks containing the complex were pooled and applied to columns of Sepharose 6B (pH 7.4, 0.01 M Tris HCl,
Fig. 3. The effect of ionic strength on the binding activity of plasma for $^{59}$Fe-EF. Plasma (0.5 ml) and $^{59}$Fe-EF (25 $\mu$l) mixtures were dialyzed in Tris HCl buffer at various concentrations of NaCl.

0.15 $M$ NaCl). Radioactivity appeared as a discrete peak immediately after the void volume and just in advance of liver ferritin added as carrier, demonstrating that the molecular size of the complex containing the $^{59}$Fe exceeded that of liver ferritin. Complex first heated at 80° C for 15 min localized in a similar fashion on columns of Sepharose 6B. These findings strongly suggest that the $^{59}$Fe is a valid label and has remained as an integral part of the EF during the experiments.

Complex formation was inhibited during starch granule electrophoresis in barbital buffer containing 4 $M$ urea. Complex is also dissociated during DEAE-cellulose column chromatography in 8 $M$ urea with phosphate gradient elution.

Fig. 4. The effect of pH on the binding activity of plasma for $^{59}$Fe EF. Mixtures consisted of 1.0 ml plasma and 200 $\mu$l $^{59}$Fe-EF. Buffers used in this experiment were: 0.05 $M$ acetate buffer at pH 4.0 and 5.0, 0.05 $M$ phosphate buffer at pH 6.0-7.0, and 0.05 $M$ barbital buffer at pH 8.0-9.0. Each point represents the average of duplicate determinations. The presence of bovine serum albumin (BSA) inhibits the non-plasma-dependent precipitation of $^{59}$Fe EF at pH values of 6.0 and below.
Characterization of the Interacting Plasma Components

Neither freezing at –20°C nor heating the plasma to 60°C reduced its binding affinity for EF. The addition of EDTA, NaCN, or sodium azide, each tested at $10^{-2}$ and $10^{-3}$ M, did not prevent complex formation. Treatment of the plasma with hydrazine or ammonium hydroxide also did not affect complex formation. Absorption of the plasma with zymosan did not eliminate its binding activity for EF. These results exclude a possible role of the plasma complement system.

Prior dialysis of the plasma in 0.01 M Tris HCl at pH 7.4, followed by a comparison of the euglobulin precipitate with the supernate, showed that EF binding activity was in the euglobulin fraction. Addition of rabbit liver ferritin to the plasma before addition of EF completely blocked the interaction, suggesting that the plasma factor is capable of binding liver ferritin as well as EF. Absorption of the plasma with spleen macrophage monolayer produced a reduction in EF binding activity. Once-adsorbed plasma lost 27.2% of its original activity, and twice-adsorbed plasma lost 41.2%.

Fractionation procedures were carried out on plasma with high EF binding activity, and individual fractions were then assayed for EF binding activity by the dialysis technique to further identify the plasma proteins involved in the complex formation with EF. Separation of plasma by starch-granule electrophoresis revealed two peaks which contained binding activity, one in the slow γ zone and the smaller one in the fast γ (or slow β) zone (Fig. 5). In plasma with weaker binding activity, the two peak heights were more nearly equal. Heating of the eluted fractions at 60°C for 30 min did not alter their binding activity for EF. Separation of the plasma on DEAE-cellulose columns also revealed two binding peaks, both characteristic for zones containing immunoglobulins, the
Fig. 6. Fractionation of normal rabbit plasma by DEAE cellulose chromatography, followed by determination of $^{59}$Fe-EF binding activity of individual fractions by the dialysis technique (lower frame). The conductivity gradient is shown in the upper frame by the discontinuous line.

Fig. 7. Fractionation of normal rabbit plasma by Sephadex G-200 gel filtration, followed by determination of the $^{59}$Fe-EF binding activity of the individual fractions by the dialysis technique (lower frame).
first containing chiefly IgG and the second immunoglobulins of more rapid electrophoretic mobility along with transferrin (Fig. 6). Gel filtration of the plasma on Sephadex G-200 demonstrated EF binding activity only in the two higher molecular weight zones, i.e., the 19S and 7S zones, with no activity in the lower molecular weight zone where transferrin emerges (Fig. 7). These fractionation studies are all consistent with EF binding by plasma immunoglobulins of both IgM and IgG classes.

**DISCUSSION**

The experiments described demonstrate an in vitro interaction between \(^{59}\)Fe-labeled EF, isolated from rabbit erythroid cells, and proteins of normal non-immune plasma. The relatively weak interaction is promoted by decreasing the ionic strength. The binding capacity of the plasma for EF is relatively low. It cannot be shown by conventional immunochemical techniques, such as immunodiffusion in agar gel, but the demonstration of complex formation was made possible by the availability of high-specific-activity \(^{59}\)Fe-EF and by techniques using relatively large volumes of plasma. From the specific activity of the EF, the binding capacity of normal plasma for EF is roughly 20 \(\mu\)g EF iron (or 180 \(\mu\)g EF protein) per 100 ml, estimated at 0.15 \(M\) NaCl concentration (Fig. 3). However, there is marked variation between different plasma specimens. The evidence suggests that the interaction is not specific for EF, since rabbit liver ferritin possessed blocking activity. The plasma factor is not species specific. The physicochemical characterization of the plasma factors which form complexes with EF suggests that they may be immunoglobulins of the IgG and IgM classes. The definitive character of the binding proteins remains to be demonstrated, however. Richter and Lee have reported the production in rabbits of an isoantibody against rabbit spleen ferritin following immunization with heterologous horse spleen ferritin, but the relation of this antibody to the plasma factors described here is not clear.

The experimental results demonstrating loss of plasma activity after adsorption by monolayers of spleen macrophages support the hypothesis that the activity represents natural antibody cytophilic for macrophages. Such a natural cytophilic antibody might promote iron salvage from the circulation or facilitate transfer through cell-to-cell interaction between erythroid precursor cells and RE cells. Evidence in favor of a physiologic role for natural cytophilic antibody was reported by Vaughan, who proposed that the interaction between macrophages and aged autologous erythrocytes is facilitated by the presence on the macrophage of a natural cytophilic antibody. The biologic significance, if any, of the EF-plasma protein interaction remains to be proven.

The direction of ferritin movement between RE cells and erythroid precursors has long been the subject of controversy. Deiss and Cartwright have reported experiments on swine reticulocytes, demonstrating a ferritin transfer pathway from the erythroid cell to plasma to monocyte. They reported that the presence of plasma was essential for the demonstration of this movement of ferritin. Possibly, the plasma factors responsible for the ferritin cell to cell exchange in the swine system are similar to those described here.
REFERENCES

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