Observations on Fe\textsuperscript{59} Labeled Bone Marrow Ferritin

By Thomas G. Gabuzda and Frank H. Gardner

With the technical assistance of Anne Council

Although ferritin has been shown to be present in many tissues of the body, the bulk of it is found in the cells of the liver, the spleen, and the bone marrow.\textsuperscript{1,2} Ferritin aggregates with other cellular constituents, it is believed, to form insoluble complexes called hemosiderin.\textsuperscript{3} Ferritin and hemosiderin constitute the body's storage depot of iron. Most of this iron supply, derived chiefly from hemoglobin breakdown, is returned to the erythroblasts for reincorporation into heme. Marrow ferritin is found both in reticuloendothelial cells as well as in erythroblasts. Whether the reticuloendothelial cell ferritin of the marrow is an anabolic source of iron for the erythroblast, or whether it is predominantly of catabolic origin, remains obscure to our understanding. Erythroblast ferritin is seen by electron microscopy both in the cytoplasm and in the cell membrane.\textsuperscript{4,5}

Marrow ferritin becomes labeled with radioiron during incubation in vitro with this isotope. This is an observation which at first glance seems self-evident, but which was only first documented in 1964 by Zail and associates.\textsuperscript{6} A polymorphism of labeled bone marrow ferritin is described in the report to follow. This polymorphism appears to be distinct from that previously described in starch gel electrophoresis.\textsuperscript{7,9}

Materials and Methods

Source of Labeled Bone Marrow Ferritin. Most of the studies reported in detail were performed on bone marrow aspirations withdrawn from the anterior iliac crest at various times over an 18-month period in a patient with chronic nonprogressive refractory sideroblastic anemia. This patient, (C. M., male, aged 56) was chosen for more intensive study because his marrow aspirates were consistently cellular, contained a large excess of stainable iron (including ringed sideroblasts), and incorporated a large fraction of radioiron into the nonhemoglobin protein fraction which contained ferritin (Fig. 1). Heparinized marrow aspirates were centrifuged to remove most of the autologous hyperferremic plasma, which was then replaced with normal type AB positive plasma previously labeled with Fe\textsuperscript{59}.
citrate or Fe\textsuperscript{59} \textit{Cl}.	extsuperscript{10} Each ml. of incubation mixture contained 0.5 to 1.0 \textmu c. of isotope and was supplemented with 1 mg./ml. glucose. Incubations of 4–6 hours duration were carried out in a Dubnoff shaker. Closed 50 ml. Erlenmeyer flasks with an air atmosphere each contained 5 ml. of incubation mixture. After completion of incubation, cells were washed 3 times in saline and hemolysates were prepared with 2 vol. water and $\frac{1}{2}$ vol. toluene. After standing overnight at 4 C., stroma was removed by centrifugation at 10,000 g for 1 hour. Subsequent studies were carried out on the supernatant.

\textbf{Separation of Nonhemoglobin Proteins from Hemoglobin.} This procedure was accomplished either by column chromatography on Amberlite CG 50 with Developer number 6,\textsuperscript{10} or by ammonium sulfate precipitation (50 per cent saturation, pH 7, at 4 C.) of the supernatant obtained after heating to 60 C. for 10 minutes. Protein fractions were concentrated when necessary against Carbowax 20M.\textsuperscript{11} The method of preparation did not affect the results.

\textbf{Preparation of Spleen Ferritin.} Spleen tissue was obtained from fresh surgical specimens and was stored frozen prior to use. Most of the spleens came from patients undergoing renal transplantation for chronic renal failure. Several methods were used to isolate ferritin from spleen tissue without noticeable effects on its properties. The basic method followed Granick’s technic,\textsuperscript{11} using fivefold cadmium sulfate crystallization, but since this method failed to produce good yields on numerous occasions, it was modified. The heating temperature was reduced to 60 C. and the cadmium sulfate eliminated; instead, purification on Sephadex G 200 followed by starch granule electrophoresis, or sucrose gradient ultracentrifugation (as described below), was used. These purification procedures were as effective as crystallization, at least as judged by the absence of nonferritin components on starch gel electrophoresis. Apoferritin was prepared from spleen ferritin by the method of Behrens and Taubert.\textsuperscript{12}

\textbf{Comparative Analyses of Bone Marrow and Spleen Ferritins.} Vertical and horizontal starch gel electrophoresis followed in general the technics of Boyer and of Poulik.\textsuperscript{13,14} Starch granule electrophoresis was done in 0.05 M veronal buffer at pH 8.6 in a closed chamber at 10 V./cm. Ultracentrifugation was done in a Spinco Model L instrument with the SW 39 rotor. The 10 to 40 per cent continuous sucrose gradient in 5 ml. tubes was subjected to 125,000 g for 2 hours.

The antiserum used in these studies was prepared against human liver ferritin in rabbits.\textsuperscript{†} With serial dilutions of the antiserum up to 1:25, it was possible to produce iron staining precipitin lines against human spleen ferritin at concentrations as low as 30 \mu g./ml. Double diffusion studies were done in 1 per cent agar gel in pH 8.3 veronal buffer. The effect of antiferritin on the electrophoretic behavior of the iron-labeled nonhemoglobin proteins was studied by incubation at 37 C. for 45 minutes of 50 \mu l. of antiferritin with a small amount of nonhemoglobin fraction diluted in saline. The specificity of the antiferritin was checked by blocking it with an excess of purified spleen ferritin in a similar incubation before its final incubation with nonhemoglobin fraction.

\textbf{Other Procedures.} Optical densities were measured in a Beckman DU spectrophotometer using cells of 10 mm. light path. Radioactivity was counted in a Packard Autogamma well-type scintillation counter. Iron staining was done with 1 per cent potassium ferrocyanide in 0.1 N HCl at room temperature following fixation. Autoradiography was performed using Ansco A-B sandwich packs with exposure periods as long as 8 months.

\section*{Results}

\textit{Proportion of Fe\textsuperscript{59} in Nonhemoglobin Fraction.} In the sideroblastic patient up to 50 per cent of the radioiron incorporated into soluble protein was found

\textsuperscript{*}Abbott Laboratories.

\textsuperscript{1}Union Carbide Company, Needham, Massachusetts.

\textsuperscript{†}Dr. John M. Craig, Department of Pathology, Boston Lying In Hospital, generously provided the antiferritin used in these studies.
in the nonhemoglobin fraction (Fig. 1). Similar patterns were seen in other iron-loading conditions, such as thalassemia and pernicious anemia. Normal bone marrow under similar conditions contained about 15 per cent of the isotope in this fraction, as others have reported.6,15 Reticulocytes from patients with sickle cell anemia or pernicious anemia during response to treatment showed even a smaller proportion of radioiron in the nonhemoglobin fraction, as Zail and his associates have found. It should be emphasized that FeS9 associated with stroma or other insoluble material, such as hemosiderin, is not taken into account in these figures.

**Immunochemical Demonstration of Ferritin in the Nonhemoglobin Fraction.** In double diffusion studies an iron stainable precipitin line showing a reaction of identity between a nonhemoglobin component and spleen ferritin could be demonstrated with ease in this patient. Autoradiography proved that this precipitin line contained radioactive iron. Such precipitin lines could be demonstrated in other patients with iron-loading anemias. However, in nonhemoglobin fractions from other types of patients stainable precipitin lines were absent. Nonetheless, their presence could sometimes be demonstrated by autoradiography as pointed out by Zail and co-workers.6 The inability to see stainable precipitin lines in these concentrated fractions suggests that the ferritin concentration is quite low.

**FeS9 Ferritin Polymorphism Not Observed in Starch Gel Electrophoresis.** Theron and associates first described that a polymer-like resolution of ferritin into multiple bands of progressively lesser intensity occurs during starch gel electrophoresis. Others have amply confirmed this observation and have shown that iron, despite its relatively greater content in the slower bands, has no in-
Fig. 2.—Vertical starch gel electrophoresis of the Fe⁵⁹-labeled nonhemoglobin fraction added to carrier spleen ferritin (left slot), the labeled nonhemoglobin fraction alone (middle slot), and of a higher concentration of spleen ferritin alone (right slot). To the right is the pattern after staining for iron (the more concentrated spleen ferritin fraction was centrifuged to remove aggregated material which caused excessive trailing). On the left is an autoradiograph of the same gel. Note that the radioactivity localizes in the same zone as the main ferritin component, and that this zone of localization is essentially independent of ferritin concentration differences.

fluence on this phenomenon, since apoferritin prepared by chemical reduction of the ferritin shows the same bands in essentially the same proportions and positions.⁷-¹⁶ Possible “native” apoferritin (that is, isolated directly from the tissues rather than artificially prepared from the previously isolated ferritin) contains only one component which corresponds to the main and most anodal component of ferritin.¹⁷ The iron-labeled nonhemoglobin protein of marrow also localizes in this zone, as shown by autoradiography of a vertical starch gel electrophoretogram (Fig. 2).

While ferritin attains the mobility of an alpha globulin in free electrophoresis, its mobility is considerably retarded in starch gel electrophoresis due to the powerful sieving effect of the gel on macromolecules. In vertical starch gel the mobility of the major ferritin component is slowed to a position only slightly
Fig. 3.—Starch granule electrophoresis of: (A) Fe$^{59}$-labeled nonhemoglobin fraction added to an excess of carrier spleen ferritin; (B) a repeat electrophoresis of the trailing shoulder of radioactivity eluted from zone A; (C) labeled nonhemoglobin fraction added to an excess of carrier spleen apoferritin; and (D) labeled nonhemoglobin fraction incubated with antihuman ferritin rabbit serum before electrophoresis. Note that the radioiron-labeled nonhemoglobin protein forms two radioactive peaks, a major peak of more rapid mobility than spleen ferritin, and a minor peak with the same mobility as spleen ferritin. Also note that if the antiferritin used in (D) was first blocked with purified spleen ferritin, then the pattern of radioactivity localization seen in (A) was restored. The symbols Alb and Hb A represent the expected mobilities of human serum albumin and of human hemoglobin A$_1$.

in front of human hemoglobin A$_1$. In the horizontal gel its mobility is still further retarded to the approximate position of human hemoglobin A$_2$. In both vertical and horizontal systems the radioiron of the nonhemoglobin fraction was always detected in a zone corresponding with the major ferritin band.

Fe$^{59}$ Ferritin Polymorphism Demonstrated in Starch Granule Electrophoresis. In this medium spleen ferritin moves as an alpha globulin at about 1.8 times the mobility of hemoglobin A$_1$. When a tracer quantity of labeled nonhemoglobin fraction was added to an excess of spleen ferritin, the radioiron in this system consistently migrated more rapidly than spleen ferritin, forming a narrow peak of radioactivity with mobility only slightly less than that of human serum albumin (Fig. 3). A tiny shoulder of radioactivity could be seen in the tail
Fig. 4.—Sucrose density gradient fractionation of: (A) Fe$^{59}$-labeled nonhemoglobin fraction alone; (B) labeled nonhemoglobin fraction added to an excess of carrier spleen ferritin; and (C) labeled nonhemoglobin fraction added to an excess of carrier spleen apoferritin. The tiny 280 m$\mu$ peak in (A) in the same position as spleen ferritin should be noted. This peak was immunochemically identified as Fe$^{59}$-labeled ferritin. Also note that the radioiron label has only slightly less density than spleen ferritin, while apoferritin is much less dense.

of this peak, falling in the same zone as spleen ferritin. When this shoulder was separated and subjected to repeat electrophoresis in the presence of carrier spleen ferritin, it localized precisely as spleen ferritin, showing that there were actually two radioactively labeled ferritin components in the nonhemoglobin fraction of this patient. One of these two components, the "slow" ferritin, appears to be the same as spleen ferritin. Iron content is apparently not responsible for this difference, since it has been reported not to influence electrophoretic mobility.$^{18}$

The identification of the two radioactively labeled components in the bone marrow fraction as ferritin was done with the use of antiferritin before electrophoresis. When this antibody was reacted with the nonhemoglobin fraction prior to electrophoresis, almost all the radioactivity was found at the origin. None was detected at the expected sites of the Fe$^{59}$ peaks. The specificity of the antiserum was verified by the fact that its activity could be blocked by prior incubation with purified spleen ferritin.

The "fast" ferritin described was certainly not unique in the sideroblastic
patient, since it was also observed as the main radioactive peak in patients with pernicious anemia, polycythemia vera and iron deficiency anemia. It also localized in the same zone in patients who were erythropoietically normal. The labeled “slow” ferritin was specifically looked for only in the sideroblastic patient, since specific activities were too low in other studies. However, in a ferritin preparation isolated from sternal marrow pooled from 3 autopsy specimens and prepared by Granick’s method, the major peak was identical in starch granule mobility to that of spleen ferritin. This indicates that “slow” ferritin of the marrow was also not unique in this patient.

Sucrose Density Gradient Fractionation. Because of its extraordinarily great iron content (up to 2400 atoms of iron/molecule), ferritin has an exceptionally high molecular density. The less iron that ferritin contains the lower its density. Because of ferritin’s heterogeneous iron content, it forms a broad, though dense, band in ultracentrifugation. Apoferritin with a molecular weight of about 460,000 has a considerably lower density than ferritin. The radioiron of the nonhemoglobin fraction behaves as a dense protein forming a band overlapping and only slightly above that of spleen ferritin (Fig. 4). It is clearly more dense than apoferritin. Since radioiron incorporated into liver ferritin is found in greater specific activity in the less dense layers, it is not surprising that the radioactive band of labeled marrow ferritin should fall slightly above the spleen ferritin color band in a density gradient. When the nonhemoglobin fraction of the sideroblastic patient was spun without added carrier ferritin, a tiny peak was demonstrated by spectrophotometric reading at 280 mµ in the same zone as spleen ferritin. This reading no doubt overestimated the relative ferritin content considerably because of the large contribution of iron to the reading at this wavelength. It emphasizes, however, that even in this iron-loaded patient ferritin appears to constitute only a very tiny proportion of the total protein of the nonhemoglobin fraction.

Discussion

A preliminary report of this work appeared simultaneously with one by Alfrey and his co-workers who used cellulose acetate and agar gel as electrophoretic media. Both reports claimed that human bone marrow ferritin exists in two forms, distinguished by their electrophoretic mobility. The “slow” ferritin has the same mobility as spleen ferritin, while the other component, “fast” ferritin, is more rapid. (The Alfrey group also reported that liver ferritin differed from both fast and slow ferritin; it showed intermediate mobility.) Although ferritin iron specific activity was not measured in the present study, “fast” ferritin contained most of the nonhemoglobin radioiron incorporated during in vitro incubation, while “slow” ferritin was seen only as a diminutive radioactive peak. Thus, “fast” ferritin may be the more active incorporator of transferrin bound iron. If the transferrin-bound iron is transported chiefly into hemoglobin synthesizing cells and if the ferritin iron of reticuloendothelial cells comes mostly from hemoglobin breakdown, the present data would then support Alfrey’s hypothesis that the “fast” strongly labeled
ferritin is characteristic of erythroblasts and reticulocytes, while the “slow” weakly labeled ferritin is from the reticuloendothelial cells.

These observations of electrophoretic differences between spleen and marrow ferritin may explain why Greenough and his associates found that the radioiron-labeled protein of the nonhemoglobin fraction of dog marrow showed a more rapid mobility than liver ferritin, and why a similar Fe\(^{59}\)-labeled component derived from rabbit red cell stroma was found by Falbe-Hansen and Lotke to move more rapidly in starch granules than rabbit liver ferritin.\(^{15,25}\)

Since the iron content of ferritin appears not to influence its electrophoretic mobility, the basis of the polymorphism just described probably lies in the protein portion of the molecule. “Fast” ferritin may be more electronegative than “slow” ferritin. However, when the two ferritins are subjected to the sieving effect of starch gel electrophoresis, the migration distance is retarded so much that the charge difference is not grossly evident. Richter has been able to find small differences in electrophoretic mobility in acrylanide gel between liver ferritin and ferritin derived from HeLa or KB cells in tissue culture.\(^{26}\)

Whether the ferritin of HeLa or KB cells is analogous to the “fast” ferritin of bone marrow is a matter for future study.

While the behavior of ferritin in starch gel electrophoresis has not as yet been proved to actually represent polymers, it remains most probable that this heterogeneity is one of molecular size, while that described in starch granule electrophoresis represents one of molecular charge. Although in the present experiments radioiron was found only in the main and most anodal of the starch gel components, further work is required to positively determine whether or not the minor components in this electrophoretic system contain Fe\(^{59}\) following marrow incubation with the isotope.

Immunochemical procedures have also suggested protein heterogeneity in the population of ferritin molecules. Both Richter and Fine and associates have stated that two and at times three precipitin lines can be seen in double diffusion studies of ferritin in agar gel.\(^{27,28}\) The authors have suggested that different ferritin types may possess unique antigenic components.

To complete the picture of ferritin heterogeneity, both ultracentrifugal analysis and fractional precipitation with ammonium sulfate indicate that its iron content is heterogeneous. The upper portion of the centrifuged ferritin band contains relatively less iron than the lower.\(^{29}\) The ferritin fractions precipitating at lower ammonium sulfate concentrations have greater iron to protein ratios than those brought down only at higher concentrations.\(^{30}\)

Thus we have suggested that four molecular dimensions of ferritin heterogeneity may be delineated: (1) molecular charge, (2) molecular size, (3) antigenic components, and (4) iron content. An important future task will be to determine the nature of the differences, how they may be related to one another, and what biological significance they may claim. There may be a genetic basis to protein polymorphism, as with the isozymes of lactic dehydrogenase, but there is no evidence as yet to support this contention for ferritin since protein polymorphism may also occur as a result of postsynthetic
changes in the molecule. The nature of ferritin polymorphism and whether it has a special relation to cellular uptake of iron from transferrin, to heme synthesis, to ferritin aggregation and iron storage, or to iron reutilization are all matters of interest which may serve as guidelines for other experiments.

SUMMARY

Qualitative studies were carried out to determine the nature of the non-hemoglobin protein or proteins of human bone marrow which incorporate radioiron during in vitro incubations of this tissue. The labeled fraction was studied chiefly by following the behavior of the radioactive iron and comparing it with ferritin isolated from human spleen. Two types of labeled ferritin were identified in the nonhemoglobin fraction. One, a “fast” ferritin, was of greater electrophoretic mobility than spleen ferritin in starch granules and appeared possibly to be the chief incorporator of transferrin-bound iron. The second, or “slow” ferritin, was identical in electrophoretic mobility with spleen ferritin and contained only a tiny percentage of the radioiron incorporated into “fast” ferritin. No other iron-labeled nonhemoglobin components were observed, although their possible presence cannot be discounted.

SUMMARIO IN INTERLINGUA

Esseva executate studios qualitative con le objectiVO de determinar le natura del proteina o proteinas non-hemoglobinic in human medulla ossee le qual incorpora radioferro in le curso de incubation in vitro de iste tissue. Le marcate fraction esseva studiata principalmente per sequer le comportamento del ferro radioactive e comparar lo con ferritina isolate ab splen human. Duo typos de ferritina marcate esseva identificate in le fraction non-hemoglobinic. Le prime, un ferritina “rapide,” habeva un plus alte mobilitate electrophoretic que ferritina splenic in granulos de amylo e pareva possibilemente esser le incorporator major de ferro ligate a transferrina. Le secunde, un ferritina “lente” esseva identic in mobilitate electrophoretic con ferritina splenic e contineva solmente un micrissime procentage del radioferro incorporate ad in ferritina “rapide.” Nulle altere non-hemoglobinic componentes marcate con ferro esseva observate, sed le possibilitate de lor presentia non pote esser negate categoricamente.

REFERENCES

Observations on Fe$^{59}$ Labeled Bone Marrow Ferritin

THOMAS C. GABUZDA, FRANK H. GARDNER and Anne Council