Bi-Directional Transport of Ferritin in Guinea Pig Erythroblasts In Vitro

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The discovery of ferritin-laden micropinocytotic vesicles in erythroblasts, the appearance of ferritin on the erythroblast surface and the invagination apparently leading to formation of micropinocytotic vesicles led Bessis to postulate that ferritin is transported into the interior of the erythroblast by this mechanism which he termed rhopheocytoxis. Since then, considerable controversy has arisen about the direction of the ferritin transport in the erythroblast and it has been suggested that ferritin may be extruded from the red cell rather than ingested by the process originally envisaged by Bessis. In a previous communication we indicated that rhopheocytoxis is most likely unidirectional into the cell, but could not exclude the possibility of loss of ferritin from the cell by some other mechanism. We have now reinvestigated the problem by two new technics: (1) the use of horseradish peroxidase to label micropinocytotic vesicles and follow their formation, and (2) in vitro study of guinea pig erythroblasts in some of which ferritin-laden micropinocytotic vesicles had been induced by hypersiderosis in order to follow the fate of ferritin. The findings to be reported confirmed the original interpretation of Bessis that rhopheocytoxis transports ferritin into the erythroblast. We have demonstrated the coalescence of micropinocytotic vesicles and their elimination from the erythroblast under the conditions studied.

Materials and Methods

Guinea pig erythroblasts were used in the basic experiments because ferritin particles on the plasma membrane of erythroblasts are readily produced in this species. This is not the case in rats or rabbits and erythroblasts from Wistar rats were used for controls in some experiments. Erythroid hyperplasia was induced by multiple injections of 1 per cent aqueous phenylhydrazine chloride for up to 14 days. No increase in the number of ferritin particles in micropinocytotic vesicles occurs after this treatment. Marrows from these animals were cultured for up to 24 hours. In some animals injections of iron dextran (Imferon, Lakeside Laboratories, Milwaukee, Wis.) were used to produce ferritin in erythroblasts and reticulocytes. Media containing 0.1–0.2 per cent horseradish peroxidase were used as described below.

Preparation of Bone Marrows for Cell Culture

Animals were killed by deep chloroform anesthesia after 0.5–1.0 ml. of heparin had been injected by means of cardiac puncture. This was done to prevent blood coagulation in the...
Marrow. Marrow from both femurs was collected in sterilized plastic petri dishes (Falcon Plastics, Beckton, Dickinson & Company, Columbus, Nebr.) which contained synthetic culture media described below. Cells were separated by repeated aspiration and expulsion of the marrow from sterilized syringes. About 20 minutes elapsed from injection of heparin to the complete preparation of both femurs from each animal. The marrows were pooled in plastic tubes and centrifuged at very low speeds (100 rpm) for 10 minutes to eliminate large particles. The cell suspensions were then washed with fresh media three times and used for culture. For 24-hour incubation the following additional steps were taken. Cell counts were adjusted to between 50,000–100,000 nucleated cells per milliliter. About 50 per cent of the cells were erythroid precursors and occurred singly or in clumps of at most four to five cells. Aliquots of about 0.5 ml. were divided in sterilized plastic tubes which contained 3 to 5 ml. of culture media. All incubations were at 37°C.

Synthetic media MEM (Monolayer-Earle's base, Cat. 11F, Grand Island Biological Co., Grand Island, N. Y.) were used. According to the manufacturer, the solution contained 10 per cent fetal calf serum in the Earle's base without glutamine. By our own analysis the media contained 17 μg. iron in 100 ml. The total iron-binding capacity (TIBC) was not measured. Analysis of the final wash solution used in the previous procedure showed the same value. For short time incubation, plain media were used. For the 24-hour culture, 1.0 ml. of 1-glutamine (Cat. 503, Grand Island Biological Co., Grand Island, N. Y.), 40,000 units of penicillin-G potassium (American Quinin, Co., New York, N. Y.) and 0.1 Gm. of streptomycin sulfate (Chase Pfizer and Co., New York, N. Y.) were added to 100 ml. of the plain culture media just before use.

Horseradish peroxidase (Type 2, Sigma Co., St. Louis, Mo.) was dissolved in the plain synthetic media to make 0.1 to 0.2 per cent solutions.

Preparation of Cells for Electron Microscopy

Incubated cells were collected by centrifugation and pellets were fixed with 0.5 to 1.0 per cent glutaraldehyde in 0.1M phosphate buffer. After washing with the same buffer, cells were treated with 1 per cent osmic acid and embedded in epoxy resin. Sections were cut with diamond knives and stained with uranyl acetate and lead compounds. Unstained sections were examined for specimens treated with horseradish peroxidase. A Phillips 300 and RCA EMU-4 were used. To analyze the number of individual ferritin particles in micropinocytotic vesicles of erythroblasts, pictures were taken at the suitable magnification (8500 times) by which whole cell images were registered in 3½” x 4” plates. The number of ferritin particles were counted under the stereomicroscope.

For the demonstration of horseradish peroxidase, cells were fixed as above. Aliquots were washed with the same buffer three times and kept in buffer solution overnight. Cells collected by centrifugation were suspended in a reagent consisting of 5 mg. of 3,3’-diaminobenzidine (Sigma Co., St. Louis, Mo.) in 10 ml. of 0.05M Tris buffer, pH 7.6, containing 0.01 per cent hydrogen peroxide. The reaction was allowed to proceed for 5 to 10 minutes at room temperature, then stopped by adding ample amounts of 1 per cent osmic acid which was replaced by a fresh batch after 10 minutes. Total fixation time with osmic acid was 60 minutes. The specimens were then washed with fresh buffer solution and reuspended in small amounts of serum from the untreated animals. They were centrifuged and solidified by glutaraldehyde. Pellets were dehydrated and embedded in epoxy resin. For controls, untreated cells were exposed to the peroxidase reagent.

RESULTS

It has been shown by others that horseradish peroxidase can be used to trace the formation of micropinocytotic vesicles in the tubular cells of mouse kidneys. We found that the same technic can be used in guinea pig erythroblasts to follow the formation of micropinocytotic vesicles. The results are described in detail for erythroblasts from guinea pigs which had been made hypersiderotic with iron dextran. Peroxidase-positive material appeared on the
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Fig. 1.—(Upper left) Guinea pig erythroblasts incubated with horseradish peroxidase. Control. Note absence of peroxidase positive material in erythroblast in center and presence of positive granules in myelocyte at left lower corner (× 8900).

Fig. 2.—(Upper right) Guinea pig erythroblasts incubated with horseradish peroxidase. One minute. Early invagination with peroxidase reaction (arrow) (× 8900).

Fig. 3.—(Lower left) Guinea pig erythroblasts incubated with horseradish peroxidase. Five minutes. Several invaginations and four vesicles of about 0.2 to 0.5 μ with peroxidase reaction (× 8900).

Fig. 4.—(Lower right) Guinea pig erythroblasts incubated with horseradish peroxidase. Thirty minutes. Similar to appearance at 5 minutes except for peroxidase positive structures attached to surface (arrows), presumably representing extrusion of vesicles (× 8900).
Fig. 5.—Guinea pig erythroblasts incubated with horseradish peroxidase. Sixty minutes. Large peroxidase positive tubular structures and positive vesicles about to rupture cell wall (arrows) (× 8900).

Surface of the erythroblasts in areas of invagination and vesicles within one minute (Figs. 1 and 2). The number of positive vesicles increased during the first 30 minutes of observation (Figs. 3 and 4). After 60 minutes confluent tubules or large vesicles formed, particularly near the surface through which they appeared to extrude in several instances (Fig. 5). A detailed study of the phenomenon suggested the sequence of events illustrated in Fig. 6. Within one minute a peroxidase positive vesicle containing ferritin could be seen to be still connected with its original invagination by a tubular structure extending to the cell surface. The tubular connection occasionally contained both ferritin particles and peroxidase (Fig. 6A). A similar pattern but with the vesicle located somewhat deeper in the cytoplasm is illustrated in Fig 6B. After five minutes (Figs. 6C, D) both slightly and heavily peroxidase positive vesicles were seen. A larger vesicle is shown in Fig. 6D. No peroxidase staining was present in vesicles of similar size belonging to the Golgi apparatus, one of which is seen at the bottom of Fig. 6C. Ferritin-laden vesicles were noted without peroxidase staining. These vesicles presumably ingested iron prior to peroxidase availability or failed to ingest the peroxidase at the same time as the ferritin. After 30 minutes much larger peroxidase positive vesicles were present (Fig. 6E), apparently originating from confluent smaller vesicles. These larger vesicles contained an abundance of peroxidase and appeared on occasion to rupture through the surface of the cell (Fig. 6F). The observations were identical in rats except that no ferritin was present. The observations during the first five minutes are consistent with the generally accepted origin of micro-
pinocytotic vesicles from invaginations and the concept that ferritin present on the red cell surface would be carried into the interior of the erythroblast in this fashion. The later confluence of these vesicles and their extrusion, however, has not been previously described. The phenomenon of extrusion was confirmed by observation of ferritin-laden erythroblasts in the absence of peroxidase from the medium. There was significant decrease in the number of ferritin particles within such vesicles when the erythroblasts were incubated for 60 minutes (Fig. 7). It was also noted that ferritin was no longer seen in invagination of the plasma membrane seen at 60 minutes of incubation. This supports the contention of the elimination of ferritin through the mechanism of extrusion of confluent vesicles.

The elimination of ferritin was further substantiated by the long term observation at 22 and 24 hours. In this experiment, erythroblasts from non-hypersiderotic guinea pigs were used. These cells contained initially very few ferritin particles. After 24 hours, many erythroblasts possessed vesicles and tubular structures which contained ferritin. Again fusion of micropinocytotic vesicles and doughnut-shaped tubular structures were noted. Some multivesicular structures were also seen (Figs. 8, 9 and 10). The structures could be interpreted as autophagic vacuoles and were considered to represent lysosomes. Such vesicles apparently ruptured and discharged smaller particles or vesicles with ferritin attached to their outside which could then be seen adjacent to the erythroblast surface. The phenomenon was observed in reticulocytes as well as in erythroblasts (Figs. 11 and 12). The similarity of these appearances to the large peroxidase positive vesicles and extrusions shown in Fig. 5 is apparent.

Because pathologic evidence for the extrusion of ferritin from the erythroblast had not previously been observed, it might be suspected that we were dealing with an in vitro phenomenon of cultured cells. Consequently, a search was made for similar appearances in freshly fixed erythroblasts or reticulocytes from hypersiderotic marrows of guinea pigs. The relevant observations are depicted in Figs. 13-16 and indicate processes identical to the extrusion just described in vitro, including multiple small vesicles with ferritin laden surfaces adjacent to an erythroblast (Figs. 15 and 16).

DISCUSSION

From the present in vitro studies of guinea pig erythroblasts, it appears that the intake of macromolecules such as peroxidase and ferritin from the environment into the cells is completed within minutes. The mechanism of rhopheocytosis carries molecules originally attached to the cell surface into the micropinocytotic vesicles within the cytoplasm. In rats, peroxidase was taken up in an identical manner, but for reasons not yet understood, ferritin is seen only exceptionally attached on the cell surface or within micropinocytotic vesicles. Confluence of such vesicles containing ferritin or peroxidase in guinea pig erythroblasts and reticulocytes into larger vesicles, or formation of multivesicular bodies (autophagic vacuoles) and elimination by rupture of these structures to the outside was observed in vitro for the first time. The process is often completed within 60 minutes. The finding of similar structures
Fig. 6.—See legend on facing page.
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Fig. 7.—Number of ferritin particles in micropinocytotic vesicle of erythroblasts from hypersiderotic guinea pigs before and after 60-minute incubation. Each dot represents one erythroblast.

![Graph showing the number of ferritin particles over time.](image)

in freshly fixed guinea pig bone marrows indicates that the elimination of ferritin by confluence and rupture of vesicles occurs in vivo as well as in vitro. One might wonder why the in vivo appearance illustrated in Figs. 13–16, which duplicate the experimentally proven extrusion, has not been described previously. Two possible explanations suggest themselves. The extruded material may be washed away rapidly in vivo. It is also possible that the extrusion mechanism may come into play only when there is an excess of ferritin as in hypersiderotic guinea pigs. The extrusion of peroxidase taken up from the environment may also be a purposeful reaction to the possibly toxic effects of peroxidase on the cytoplasm.

The observations provide direct morphologic proof of elimination of iron

Fig. 6.—Guinea pig erythroblasts incubated with horseradish peroxidase. Possible sequence of vesicle turnover. (A) Early stage of membrane invagination and vesicle formation engulfing ferritin particles attached to surface (× 5700). Note vesicles with negative peroxidase reaction containing ferritin (arrow). (B) Fusion of ferritin-containing tubule with nonferritin-containing vesicle (× 5700). (C) Peroxidase positive vesicle with ferritin (arrow near Golgi apparatus). Note absence of peroxidase reaction in Golgi vesicle below (× 8200). (D) Peroxidase positive vesicle of about 0.3 μ (× 5900). (E) Positive vesicle of about 0.5 μ containing positive rings of about 80 mμ (arrows) (×5900). (F) Vesicle of similar size rupturing cell membrane (× 5900).
Fig. 8.—Reticulocytes and erythroblasts incubated in vivo without peroxidase. Various stages of ferritin collection in vesicles (arrows). Note formation of small secondary vesicles within them (× 60,000).

Fig. 9 (Left)—Reticulocytes and erythroblasts incubated in vitro without peroxidase. Ferritin particles collected in large vacuole containing small vesicles and electron opaque ground substance (× 68,000).

Fig. 10.—(Right)—Reticulocytes and erythroblasts incubated in vitro without peroxidase. Inclusion of type similar to Fig. 9 with more distinct secondary vesicles (× 68,000).
from the erythroblasts as postulated by Jandl. The observation may be related to the elimination of iron from swine siderocytes with concomitant increase of iron in the culture medium reported by Deiss and Cartwright. Evidence that elimination of iron-containing autophagic vacuoles occurs in the spleen of man was adduced by Kent et al. who found the number of such vacuoles markedly increased in splenectomized patients. The mechanism would explain the observation of Crosby who transfused siderocyte-rich blood labeled with $^{51}$Cr from splenectomized patients into recipients with intact spleens. The red cells were shown to survive after having been depleted of iron granules, presumably in the spleen.

The present observation that the amount of ferritin in and around guinea-pig erythroblasts from non-hypersiderotic marrows increases during 24 hours of incubation is compatible with an earlier suggestion that erythroblasts may synthesize ferritin on its surface. There is considerable evidence that erythroblasts can synthesize ferritin but the postulated synthesis of ferritin on the erythroblast surface requires further confirmation. It may be specific for guinea-pigs and man since only in those species has an increase in surface ferritin been demonstrated in hypersiderosis.

**SUMMARY**

Direct morphologic evidence has been presented for a bi-directional flow of ferritin in guinea pig erythroblasts and reticulocytes both in vitro and in vivo. Ferritin intake by means of micropinocytotic vesicles is followed by their
Fig. 13 (Top left)—Erythroblasts and reticulocyte in bone marrow fixed without incubation. Vacuole containing small vesicles and ferritin analogous to appearance in cultured cells (× 68,000).

Fig. 14 (Middle left)—Erythroblasts and reticulocyte in bone marrow fixed without incubation. Elimination of ferritin from medium-sized vesicle (× 100,000).

Fig. 15 (Bottom left)—Erythroblasts and reticulocyte in bone marrow fixed without incubation. Elimination of ferritin and vesicles from reticulocyte. Note similarity to appearance of cultured cells in Fig. 12 (× 64,000).

Fig. 16 (Right)—Erythroblasts and reticulocyte in bone marrow fixed without incubation. Collection of ferritin particles in vesicles of up to 0.5 μ, some with secondary vesicles (large arrows). Eliminated ferritin adherent to secondary vesicle (small arrows). Large ring structure containing ferritin is seen extracellularly (asterisk) (× 66,000).
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confluence and rupture of the large vesicles to the outside within 60 minutes. The origin of ferritin on the red cell surface prior to its uptake by the micro-pinocytosis (rhopheocytosis) is yet to be determined.

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REFERENCES

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