Heinz Body Anemia: An Ultrastructural Study. II. Red Cell Sequestration and Destruction

By RICHARD A. RIFKIND

THERE IS REASON to believe that a clue to the mechanisms whereby the spleen and other portions of the reticuloendothelial system exercise their function in the selective removal of injured red blood cells may reside in the particular anatomical arrangements of the phagocytic and vascular components of these organs. In order to visualize, at the ultrastructural level, the process of cell sequestration and destruction as it occurs in the spleen and liver, the hemolytic anemia induced by phenylhydrazine was selected as a model. Red cells injured by this drug undergo a series of structural alterations involving both hemoglobin and the plasma membrane which permit definitive identification of the injured cells during their residence in the reticuloendothelial organs. The present studies describe the disposition of drug-injured erythrocytes during splenic and hepatic sequestration. Several mechanisms which may contribute to this process are elucidated. The intracellular fate of red cell components following erythrophagocytosis is observed and evidence is presented for existence of at least two mechanisms of hemolysis in this anemia.

MATERIALS AND METHODS

Rabbits were injected intramuscularly with phenylhydrazine in single doses of from 5 to 15 mg./Kg. of body weight or 5 mg./Kg. on 3 consecutive days. The animals were sacrificed at appropriate intervals, by ether inhalation and the livers and spleens removed. Small fragments of both organs were fixed in cold 1 per cent glutaraldehyde in 0.067 M phosphate buffer for 1 hour, washed, post-fixed in oxmium tetroxide, dehydrated and embedded in epoxy resin. Thin sections were stained with uranyl acetate, lead citrate, or both, and examined in an RCA EMU 2E electron microscope. Hemoglobin measurements and the enumeration of Heinz body-containing red cells in the peripheral blood were performed by standard technics. Plasma hemoglobin levels were estimated by the method of Crosby and Furtth.

RESULTS

Spleen

The overall ultrastructural architecture of the red pulp of rabbit spleen, as observed in these experiments, confirms the observations of Weiss and of Roberts and Latta. Both splenic sinusoids and cords were readily distinguished by the criteria established by these authors. The morphology of the cordal and sinusoidal lining cells, however, could not be easily categorized into the...
various types which have been described,7 most likely because of the cellular reaction of the spleen in response to the brisk hemolysis. The intensity of this cellular reaction was proportional to the amount of phenylhydrazine administered and was accompanied by considerable mitotic activity, as suggested previously by Azen and Schilling.8 The increased cellularity associated with the larger doses of phenylhydrazine was largely confined to the cordal regions.

During the first 24 hours following small single doses of phenylhydrazine (5 and 10 mg./Kg.) the rabbits developed from 50 to 60 per cent of Heinz body-containing red blood cells in their peripheral blood and a very mild anemia. Their spleens displayed morphologic evidence of selective sequestration of drug-injured red cells. These cells, identified by their content of Heinz bodies,3 were observed in large numbers in the Billroth cords, exceeding by far their proportion in the peripheral circulation. In the rabbit whose spleen is illustrated in figure 1, nearly all red cells within the cordal spaces contained Heinz bodies, whereas only 50 per cent of the circulating erythrocytes bore this lesion.

Observations on the appearance and disposition of red cells in the splenic red pulp help to elucidate mechanisms which may account for or contribute to the process of sequestration. Within the highly cellular cordal vascular spaces red cells are squeezed as they pass between adjacent lining cells and free-lying macrophages. Likewise, upon passing from cords into the sinusoids, the erythrocytes are tightly compressed between cellular elements and the fenestrated basement membranes. Uninjured erythrocytes become strikingly attenuated as they pass through such narrow channels. Heinz bodies, however, which consist of insoluble denaturation products of hemoglobin,9 seem to be relatively nondeformable structures, as suggested by the severe deformation of cell shape which they produce.3 Figure 2 illustrates a drug-injured, Heinz body-containing red cell in the splenic red pulp. That portion of the red cell containing predominantly native hemoglobin has passed through a fenestration in the cordal wall into a sinusoid. The Heinz bodies, however, have collected on the proximal (cordal) side of the basement membrane and appear to have restricted further progress of the red cell out of the cordal space.

There occurs, in the splenic cords, in addition to intravascular sequestration, an active erythrophagocytosis, in which both endothelial lining cells and free-lying macrophages participate. The vast majority of the ingested red cells contain Heinz bodies. As illustrated in figure 3, the protruding Heinz body appears to provide the initial purchase for the macrophage in the process of trapping and phagocytizing the red blood cell. Red cells are engulfed whole, or in part, and the final destruction of the cell takes place within the macrophage cytoplasm. Early phases of this process are seen in figure 4. This macrophage has ingested several red cells which have come to lie within phagocytic vacuoles. The red blood cell at lower left (labeled 1) appears virtually intact and contains a density of hemoglobin comparable to that of a circulating red cell. It is probably the most recently ingested of the 3 red cells in this field. The middle red cell (labeled 2) displays a moderate...
Fig. 1.—Billroth cord of the red pulp of a phenylhydrazine-treated rabbit. The cordal space is partially divided by a reticulum (ret) consisting of a basement membrane and attenuated cordal lining cells. Free-lying macrophages (m) and a large number of Heinz body-containing red cells (RBC) fill the cordal spaces. A Heinz body is indicated with an arrow. Mag. x 9000.
loss of hemoglobin concentration, while the red cell at upper right (labeled 3) is only a ghost, composed of plasma membrane and Heinz bodies. It seems likely, but unproved, that native hemoglobin is, in large part, digested into small, diffusible fragments within the ingested red cell by enzymes produced by the macrophage (see Discussion). The denatured hemoglobin derivatives constituting the Heinz bodies seem more resistant to digestion, perhaps because of their relative insolubility (figs. 4, 5 and 6). The red cell plasma membrane remains at least grossly intact until after the native hemoglobin is dispersed (fig. 5). Subsequently, this membrane also disintegrates (fig. 6) within the phagocytic vacuole.

The macrophage displays a number of changes which may be considered as reactions to the injured and ingested red cell. Most striking is the proliferation of membranes. The membranes of phagocytic vacuoles which contain red cell
**Fig. 3.—Portion of a splenic cord.** The septal reticulum passes through at the lower left. Small dense bodies of an undetermined nature lie within the basement membrane. The cytoplasm of a large macrophage occupies most of the field. Early stages in the phagocytosis of several adjacent red blood cells is suggested by the engulfment of their protruding Heinz bodies (hb). A fully ingested, but still intact erythrocyte is indicated (RBC). Mag. $x$ 23,000.

Heinz body ghosts are frequently reduplicated and assume the appearance of myelin-figures (fig. 5, insert). Numerous congeries of membranous material (fig. 5, labeled m) probably represent the remains of prior erythrophagocytoses. The smooth-membrane Golgi system of vesicles and tubules is enlarged in these active phagocytes (fig. 6), and bears a close topographic relationship to the membranes of the phagocytic vacuole.

In addition to membrane formation, accumulation of ferritin is another characteristic of the erythrophagocyte. The iron-micelles of this molecule are
Fig. 4.—A macrophage in which 3 ingested red cells display apparently sequential stages in the digestion and extraction of hemoglobin. The red cell at the left (labeled 1) is virtually intact. Cell 2 displays partial loss of hemoglobin, while cell 3 consists only of a plasma membrane and residual Heinz bodies. The macrophage nucleus is labeled n. Mag. x 15,000.

generally seen throughout the macrophage cytoplasm, both within and without the phagocytic vacuole (fig. 5, insert). After long periods of hemolysis, ferritin accumulates in large, tightly packed vacuoles and bodies, an early form of which is seen in figure 5. These are often located within masses of material which appear to represent residual red cell debris. During the period of subsiding hemolysis, a week or more after the injection of phenylhydrazine, evidence for active cordal sequestration is scarce. On the other hand, many macrophages display the residua of their past erythrophagocytic activity, consisting of coalesced remnants of Heinz bodies and deposits of ferritin (fig. 7).

Especially common in the splenic cords after larger doses (15 mg./Kg.) or protracted courses of phenylhydrazine are Heinz body-containing cells which display a moth-eaten appearance and swollen contours suggestive of spherocytosis (fig. 8). It is suggested that such cells have sustained severe osmotic alterations. No evidence was obtained to suggest that injured red cells of this kind were phagocytized in any significant numbers by cordal macrophages. Observations on the splenic sinusoids, however, do provide an indication of the fate of these erythrocytes.
Fig. 5.—Remnants of an ingested red cell within a phagocytic vacuole. Most of the native hemoglobin has been extracted while denatured Heinz body material persists at the red cell margin. Evidences of prior erythrophagocytoses are the collections of membranes (m) and residual Heinz body material (hb). One such remnant contains a deposit of ferritin (f). The macrophage nucleus is seen at the lower right. Mag. x 23,000. The insert shows the region indicated by an arrow at higher magnification. The red cell plasma membrane (pm) appears intact and distinct from the reduplicated membranes of the phagocytic vacuole (v). The iron micelles of randomly distributed ferritin molecules are seen as fine, dense particles within and outside the phagocytic vacuole. Mag. x 75,000.
Fig. 6.—A more advanced stage of red cell digestion. The plasma membrane is partially fragmented (arrows). An elaborate Golgi apparatus (g) is seen in the macrophage cytoplasm. Mag. x 23,000.

The splenic sinusoids constitute relatively open vascular channels as compared with the highly cellular cords. The proportion of Heinz body-containing erythrocytes is lower than in the cords and more closely approaches that of the circulating blood. Hemolyzed ghosts of Heinz body-containing red cells are very frequently observed in the extracellular compartment of the splenic sinusoids of rabbits which have received either the larger single doses of phenylhydrazine (15 mg./Kg.) or repeated daily doses (fig. 9). These bits of red cell stroma, which are virtually never observed extracellularly within the splenic cords, are probably the result of intravascular hemolysis. The cells most likely to undergo intravascular lysis are the osmotically deranged, sphered cells observed in the cordal spaces (fig. 8). Such cells may escape erythrophagocytosis while in the splenic cords, but lyse, either spontaneously, or due to mechanical trauma, during or after passage into the sinusoids. The stromal debris which they leave is cleared from the sinusoids by macrophages which trap and ingest the ghosts (fig. 10). Plasma hemoglobin levels of 50 mg. per cent or more confirm the intravascular site of a proportion of the hemolysis in rabbits given these large doses of the drug.

Liver

Rabbits receiving single 5 mg./Kg. doses of phenylhydrazine displayed no evidence of red cell sequestration or destruction in their liver sinusoids. However, raising the dose of drug administered was accompanied by increasing evidence of erythrophagocytosis by Kupffer cells. The process of red cell
Fig. 7.—Part of a cordal macrophage a week after phenylhydrazine administration. Residua of ingested Heinz bodies are numerous. All contain ferritin, some in large amounts (arrows). Part of the cordal reticulum extends across the top of the micrograph. Mag. x 23,000.

digestion was indistinguishable from that observed in the spleen. In the liver, however, no evidence was obtained for a significant extracellular, cells-sequestering vascular compartment comparable to the splenic cords. Injured red cells bearing Heinz bodies were observed only within the phagocytic cells. No intravascular red cell ghosts were seen in this organ.

DISCUSSION

A nice discrimination exists in the reticuloendothelial system (RES), whereby moderately injured red blood cells are predominantly removed from the circulation by the spleen while more severely damaged cells are handled
Fig. 8.—A spherred, and probably prelytic, Heinz body-containing red cell (sph) adjacent to a portion of the cordal reticulum. Part of an apparently normal erythrocyte is seen at the lower right. Mag. x 14,000.

by the reticuloendothelial systems as a whole, with the liver contributing the major component by virtue of its size and blood flow. Azen and Schilling have demonstrated that this relationship of degree of damage to site of sequestration applies, as well, to phenylhydrazine-damaged red cells.

The morphology of the RES of the liver and spleen provides some reasonable mechanisms for the selection of moderately or severely damaged erythrocytes exercised by these organs. The intermediate circulation of the spleen, on the one hand, commits arterial blood to a passage through the highly cellular and compartmentalized cordal zone before emptying it into the sinusoids and the venous circulation. The present studies demonstrate intense and selective sequestration of drug-injured red cells within the vascular spaces of the splenic cords. One function of the cords, as a mechanical filter retarding the passage of Heinz body-containing cells and prelytic, spherred erythrocytes, is
apparent from the present studies, and in agreement with the experimental model of Jandl et al.21 There is presumptive evidence from other studies that the cords function, as well, as a kind of selective “column,” recognizing alterations in the physicochemical characteristics of the red cell surface.22,23 The plasma membrane injury sustained by drug-injured red cells22 and the adhesive interaction of macrophages and effete erythrocytes24 may be adduced as evidence for the latter hypothesis. Whichever mechanism predominates, clearly, in the spleen, there is a primarily extracellular, intravascular phase of red cell sequestration, as suggested previously by Jandl and co-workers.21

In the liver, on the other hand, as well as for the bulk of the extrasplenic RES,25–28 vascular continuity is achieved by way of relatively wide sinusoids lined by macrophages and endothelial cells. The present studies fail to find evidence in the liver for any significant intravascular filtration compartments comparable to the Billroth cords. The primary process of sequestration appears to involve erythrophagocytosis, presumably preceded by the adhesion of red cell to macrophage. Whereas sequestration and erythrophagocytosis is observed in the spleen after low doses of phenylhydrazine, it is only after the larger or repeated doses that hepatic macrophages display activity. Azen and Schilling20 also have demonstrated hepatic uptake after large doses of drug and indicate that this is not due to saturation of the splenic macrophages. That the degree of red cell injury is a dose-related function is suggested by kinetic data8 and by the biochemical observations of Jandl et al.9 It is not unlikely,
Fig. 10.—Lumen of another splenic sinusoid within which are residual lysed and partially lysed red cell ghosts which contain Heinz bodies (lower right). A sinusoidal macrophage (m) has ingested some of this red cell debris. Mag. x 23,000.

therefore, that for red cell-macrophage interaction to occur, under the conditions of relatively unimpeded flow provided in hepatic sinusoids, requires considerably more damage to the erythrocyte than is demanded by the spleen. Taken as a whole the present data and the observations of Weiss1,2 suggest that the fine selectivity exercised by the spleen and liver for red cells which have sustained different degrees of injury, resides primarily in the vascular anatomy of their reticuloendothelial components.

In the spleen the second phase of red cell sequestration is the actual destruction of injured erythrocytes. At least two mechanisms for this process are demonstrated by the present investigation. Most prominent is erythrophagocytosis of cells trapped within or passing through the splenic cords. Active splenic erythrophagocytosis following drug-induced red cell injury has been described on several occasions.29,30 Protruding Heinz bodies are often the first portion of the red cell attacked by the macrophage. Whether this is
due to mechanical factors or is related to the membrane injury observed at these points is not clear. The injured red cells are ingested whole and lysis occurs within the macrophage cytoplasm, confirming the interpretation of Rothberg et al. Essner has noted the presence of acid phosphatase activity in erythrophagocytic vacuoles, suggesting that these bodies are lysosomes, presumably capable of the hydrolysis of red cell components including hemoglobin. Native hemoglobin is most rapidly degraded and removed from the erythrocyte while the insoluble Heinz bodies and cell membranes are more gradually destroyed. There is a striking proliferation and reduplication of intracytoplasmic membranes, often in close association with an expanded Golgi apparatus, in the cordal macrophages. It is likely that this is one morphologic manifestation of the enhanced incorporation of lipoprotein precursors which accompanies phagocytosis. Eventually the splenic cords become pock-marked with the residua of erythrophagocytosis, including Heinz body remnants, myelin-like figures and ferritin deposits.

A second and probably quantitatively less significant mechanism of red cell destruction entails the intravascular, intrasplenic lysis of injured red cells. Certain Heinz body-bearing erythrocytes, as previously reported, display evidence of increased osmotic fragility, manifested by sphering and hemoglobin dilution. Weed et al. have provided evidence for a disturbance of ion transport in drug-injured erythrocytes which may be a forerunner of the grosser loss of osmotic stability apparent in sphered red cells. It is possible, as suggested by Griggs et al. in the case of congenital spherocytosis, that stasis and sequestration in the spleen itself results in an increased osmotic fragility of some defective red cells. Hemolysis may be spontaneous, but the mechanical trauma attendant upon splenic passage probably contributes to the process. The debris resulting from intravascular hemolysis, consisting of cell membranes and residual Heinz bodies, is cleared from the circulation by macrophages of the splenic sinusoids.

In conclusion, the choice of phenylhydrazine-induced hemolytic anemia as a model system for morphologic study appears fortunate as it is possible to demonstrate manifestations of a variety of reticuloendothelial functions. The spleen behaves as a mechanical filter for certain cells and, perhaps, as a selective impediment dependent upon surface injury for others. Both factors lead to red cell stasis, progressive injury and enhanced erythrophagocytosis. Still other red cells, perhaps due to their advanced osmotic instability, lyse within the vascular compartment. The differential susceptibility to splenic or hepatic sequestration of red cells which have sustained varying degrees of injury appears to result not from an inherent difference in macrophage potentialities, but from the peculiar anatomical arrangements of the splenic vasculature.

**Summary**

This study reports electron microscope observations on the process of red cell sequestration and destruction in the spleen and liver of the phenylhydrazine-treated rabbit. Damaged red cells are recognized by virtue of
their Heinz bodies, a morphologic manifestation of the oxidative injury which they have sustained. Sequestration, in the spleen, involves the selective accumulation of damaged cells within the vascular spaces of the Billroth cords. Erythrophagocytosis and the intracellular digestion of red cells follows sequestration. More severely injured cells may undergo intravascular hemolysis within the splenic red pulp. In the liver, however, no evidence for the intravascular sequestration of injured red cells is observed. Damaged cells are removed directly from the sinusoidal blood by erythrophagocytosis. The selectivity of spleen and liver for red cells subjected to different degrees of injury is discussed in terms of the observed differences in the vascular architecture of the two organs.

SUMMARIO IN INTERLINGUA

Le presente studio reporta observationes effectuate per medio del microscopio electronic concernente le processo del sequestration e destruction de erythrocytos in le splen e le hepate de conilios tractate con phenylhydrazina. Lesionate erythrocytos es recognoscibile per le presentia de corpores de Heinz, le quales representa un manifestation morphologic del traumatismo oxydative que illos ha suffrite. Sequestration in le splen consiste in le accumulation selective de lesionate cellulas intra le spatios vascular del cordas de Billroth. Erythrophagocytose e le digestion intracellular de erythrocytos seque le sequestration. Plus severemente lesionate cellulas pote suffrer hemolyse intravascular intra le pulpa rubie del splen. Del altere latere, in le hepate nulle evidentia pro le sequestration vascular de lesionate erythrocytos es observate. Lesionate cellulas es eliminate directemente ab le sanguine sinusoidale per un processo erythrophagocytotic. Le selectivitate de splen e hepate pro erythrocytos subjicite a varie grados de traumatismo es discutite con respecto al observate differentias in le architectura vascular del duo organos.

ACKNOWLEDGMENT

This work was supported by a grant from the U. S. Public Health Service (AI 05493). Doctor Rifkind is the recipient of a Career Scientist Award o’ the Health Research Council of the City of New York under contract No. I-336.

The author is indebted to the Department of Microbiology of Columbia University and, in particular to Doctor Councilman Morgan, for making available the electron microscope laboratory. We are grateful to Doctor Paul A. Marks for many helpful suggestions. Miss Hazel Zwack provided expert technical assistance.

REFERENCES

HEINZ BODY ANEMIA

30. Cruz, W. O.: Acetylphenylhydrazine anemia. I. The mechanism of eryth-
rocyte destruction and regeneration.
Heinz Body Anemia: An Ultrastructural Study. II. Red Cell Sequestration and Destruction

RICHARD A. RIFKIND