Virchows Arch. A Path. Anat. and Histol. 374, 339–351 (1977)

Virchows Archiv A

An Ultrastructural Study of the Red Pulp of the Spleen and the Liver in Unstable Hemoglobin Hemolytic Anemia*

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Summary. Electron microscopic observations on the process of red cell destruction in the spleen and liver of a patient with congenital Heinz body hemolytic anemia, associated with a new variant of unstable hemoglobin, are reported. Two major mechanisms of destruction of Heinz body-containing red cells were noted. One was phagocytosis of these cells in toto by cordal macrophages. The other mechanism, though less significant quantitatively, was intravascular hemolysis of injured red cells in the splenic microvasculature. In the liver, phagocytosis of damaged red cells by Kupffer cells was rare and there was no evidence of intravascular hemolysis in this organ. These morphological findings, together with almost complete recovery from hemolysis following splenectomy, indicated that Heinz body-containing red cells were removed from the circulation predominantly by the spleen. In contrast to experimental Heinz body anemia in animals, Heinz bodies were present even in the nucleated red cells.

Key words: Spleen – Liver – Heinz bodies – Unstable hemoglobin.

Introduction

The behavior of the spleen in experimental Heinz body hemolytic anemias in animals has been well documented (Rothberg et al., 1959; Koyama et al., 1964; Rifkind, 1965; Schnitzer and Smith, 1966; Lawson et al., 1969; Chen and Weiss, 1973; Leblond, 1973; Klausner et al., 1975). At least two major mechanisms of hemolysis, namely, erythrophagocytosis and intravascular lysis of injured red cells in the spleen, have been described in phenylhydrazine hemo-

* Supported by a Research Grant for Specific Diseases from the Ministry of Health and Welfare and a Grant-in-Aid for Scientific Research of the Ministry of Education, Japan

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lytic anemia in rabbits (Rifkind, 1965). The "pitting" function of the spleen, which refers to its ability to remove solid particles from the cytoplasm of a red cell without destroying the cell itself, is also known to be an important mechanism in removing Heinz bodies (Crosby, 1959; Koyama et al., 1964). Based on electron microscopic observations, Leblond (1973) and Klausner et al. (1975) have suggested an additional mechanism in which the spleen acts as a simple filter.

In this study, we describe the fine structure of the red pulp of the spleen and the liver from a patient with unstable hemoglobin hemolytic anemia and present ultrastructural evidence concerning the splenic clearance of Heinz body-containing red cells.

Case Report

A patient, 3-year-old girl, was admitted to the Nagoya City University Hospital in August, 1975, with the chief complaints of anemia, jaundice and splenomegaly. She was born following normal pregnancy in January, 1972. There was no family history of hematologic disorders, jaundice or consanguinous marriage. She had severe neonatal jaundice and was treated by phototherapy with beneficial effect. Jaundice became prominent at one month of age and she was then found to have a hemolytic anemia. From the age of eight months she had required repeated blood transfusions.

On admission, the patient was poorly developed; physical examination revealed pallor, jaundice, splenomegaly (5 cm below the left costal margin) and hepatomegaly. Laboratory data disclosed a hematocrit of 27%, a hemoglobin of 8.2 g/100 ml, a reticulocyte count of 44.1%, and a total bilirubin of 11.4 mg/100 ml serum. The stained film showed moderate aniso-poikilocytosis and basophilic stippling of red cells (Fig. 1), but Heinz bodies were not observed. Erythrocyte osmotic fragility in fresh blood and blood incubated for 24 h was not increased. Autohemolysis after 48 h incubation at 37° C was slightly increased (11% hemolysis without added glucose and 3.8% hemolysis

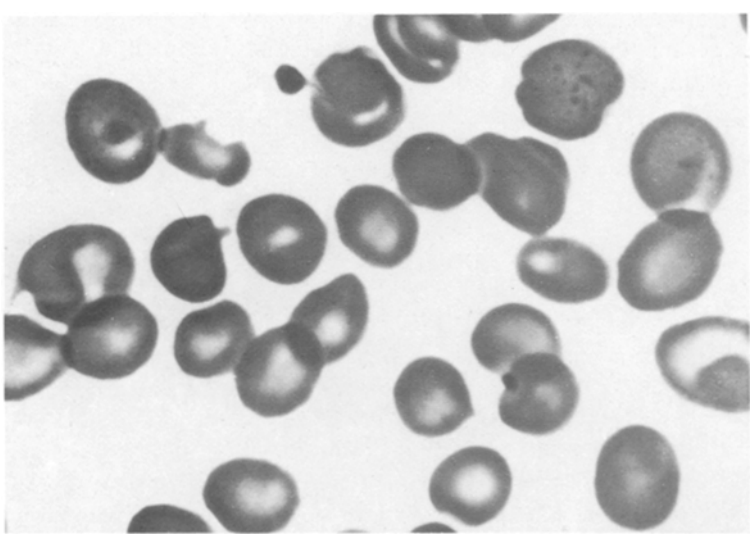


Fig. 1. A blood smear before splenectomy shows moderate aniso-poikilocytosis and basophilic stippling. Wright stain ×3300

with added glucose). Red cell enzyme studies and determination of glycolytic intermediates and adenine nucleotides showed a slight increase in all enzyme activities and in ATP levels, which were attributed to a disproportionate number of younger cells. The survival of patient's ^{51}Cr-tagged red cells after autologous transfusion was shortened, with a half-life of 6 days. She continuously passed brown-colored urine.

The patient's hemoglobin was positive for heat and isopropanol precipitation tests. Further chemical characterization of this unstable hemoglobin proved it to be a new variant that was named Hb Mizuho (β 68 Leu-Pro) (Miyaji and Shibata, 1976). This unstable hemoglobin was not demonstrated in hemolysates from her father and mother.

Splenectomy was performed in January, 1976, when she was four years of age. The spleen weighed 350 g and a small accessory spleen measuring 1 cm in diameter was also removed. Following the operation red cells containing Heinz bodies were noted in the peripheral blood, but further transfusions were not required. Laboratory data at four months after splenectomy showed a hematocrit of 36.0%, a hemoglobin of 11.5 g/100 ml, and a total bilirubin of 3.2 mg/100 ml serum with a direct bilirubin of 1.2 mg/100 ml serum. At the time of this study (December, 1976), she is leading a normal life.

Materials and Methods

For electron microscopy, small tissue fragments from several different sites of the spleen were immediately fixed in 4.15% glutaraldehyde in cacodylate buffer at pH 7.4 for 2 h at 4°C. After post-fixation in 1% OsO_4, the specimens were dehydrated in a series of alcohols and embedded in epoxy resin by the method of Luft (1961). The liver and an accessory spleen obtained at the time of operation were similarly treated. Thin sections cut with an Ivan Sorvall IIb ultramicrotome were doubly stained with uranyl acetate and lead citrate and examined with a Hitachi HS-8 electron microscope.

Thicker sections from epoxy resin embedded blocks were stained with toluidine blue for light microscopic examinations.

Larger fragments of the spleen, accessory spleen and liver were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin and Prussian blue reaction for the demonstration of iron pigment.

Results

The spleen was markedly congested. The cut surface was dark red in color and lymphoid follicles were hardly discernible. There was no evidence of infarction.

Light Microscopy

The cords of Billroth were markedly congested and had an increased number of large macrophages which contained abundant iron pigment. The sinuses were not distended and contained varying numbers of red cells. A layer of erythrocytes attached to the inner surface of the sinuses suggested retardation of these cells in their passage through the sinus walls. The red cells retained within sinus walls revealed tear-drop deformation and those in the sinus showed marked variation in size and shape (Fig. 2).

The white pulp was atrophic. There was an increased deposition of intracellular hemosiderin pigment throughout the red pulp. Iron pigment was also deposited in the basement membranes of the sinus wall and in the trabeculae.

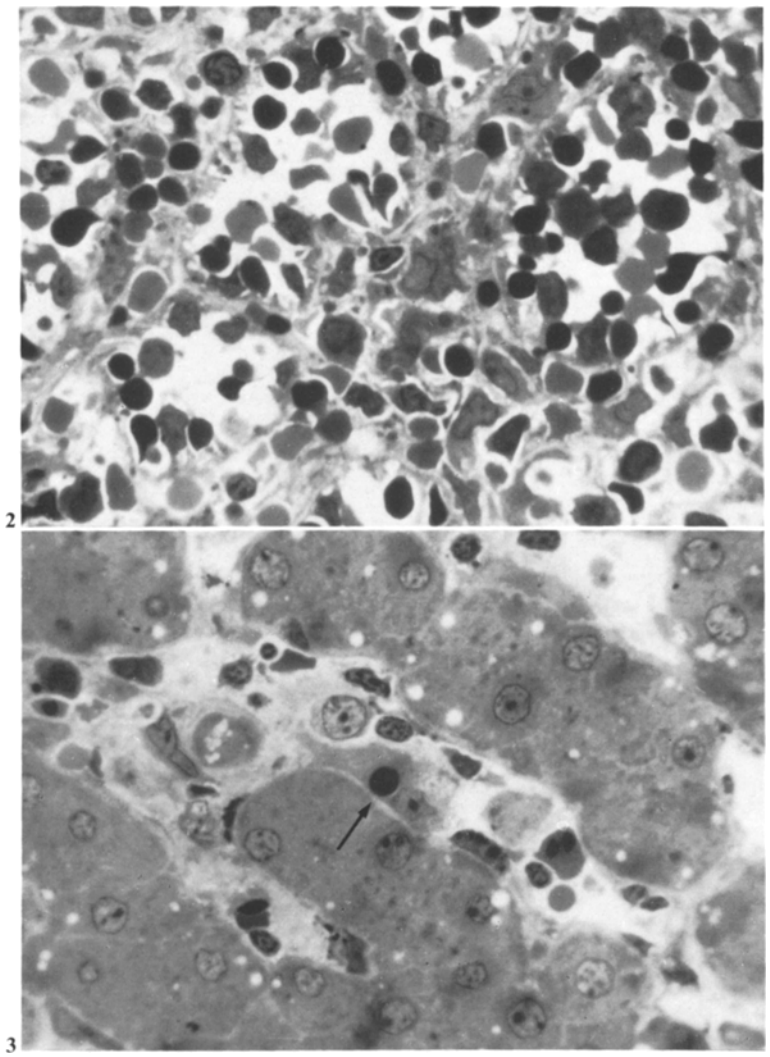


Fig. 2. Light micrograph of a section of epoxy resin embedded spleen stained with toluidine blue. Most of the red cells attached to the sinus wall reveal tear-drop deformation. ×1800

Fig. 3. Erythrophagocytosis by a Kupffer cell (arrow). Toluidine blue stain ×1800

The accessory spleen showed essentially similar morphology, except for pronounced proliferation of collagen fibers in the red pulp.

In the liver, deposition of brownish pigment was prominent, not only in the Kupffer cell but in the hepatic parenchymal cell as well. Some of the pigment deposits were negative for Prussian blue reaction. Erythrophagocytosis by Kupffer cells was only occasionally found (Fig. 3).

Extramedullary erythropoiesis, manifested by an accumulation of several or more nucleated red cells, was noted in the spleen and liver.

Electron Microscopy

Widened and congested cords of Billroth were filled with red cells which contained varied number of Heinz bodies (Fig. 4). Significantly more Heinz body-containing red cells were present in the cords than in the sinuses, indicating the difficulty of these cells in passage through the cords into the sinuses.

Two main mechanisms in which Heinz body-containing red cells were destroyed within the spleen were noted. Phagocytosis of Heinz body-containing red cells by the cordal macrophages was frequently observed. The early phase of erythrophagocytosis was represented by Heinz body-containing red cells which were almost completely surrounded by the cytoplasmic processes of macrophages, showing engulfment of such cells in toto. Recently phagocytized red cells were surrounded by a single membrane within the cytoplasm of macrophages and revealed minimal morphological changes (Fig. 5). As the intracellular degradation progressed, the hemoglobin disappeared from the red cell and only Heinz body material was seen within the collapsing membranous structures (Fig. 6). In the advanced stage of digestion, red cell membranes disappeared and Heinz body material was condensed into dense granules.

Heinz body-containing red cells which escaped phagocytosis by the cordal macrophage were trapped during their passage across the sinus wall. The major portion of erythrocytes with or without a few small Heinz bodies were lying within the sinus lumen, while portions containing most of the Heinz bodies remained in the cord. These two portions of the cell were connected by a thin membrane stalk between the endothelial cells of the sinus. In some areas, the Heinz body-containing portion was surrounded by the cytoplasmic processes of a macrophage indicating selective elimination of Heinz bodies (Fig. 7). Not infrequently narrow stalks of red cell membrane revealed breaks as shown in Figure 8. The presence of free Heinz body fragments in the cordal region indicated that this process of pitting of Heinz bodies occured at the sinus wall. It was assumed that the process of fragmentation was followed, sooner or later, by phagocytosis of Heinz body fragments by the cordal macrophage and the release of fragile cells into the sinus.

Another major process of destruction of affected red cells was intravascular hemolysis in the splenic microvasculature. Ghosts of hemolyzed red cells were noted in the cord and sinus. Most of red cell ghosts in the cord contained varying numbers of Heinz bodies (Fig. 9), while those in the sinus consisted of membrane-bound sacks usually containing only a few Heinz bodies. The latter represented the ghosts of severely damaged red cells produced by previous pitting of Heinz bodies. This intravascular lysis of affected red cells was less significant since hemolyzed ghosts were infrequently seen in the extracellular compartment of the splenic red pulp.

Another finding of interest was the presence of Heinz bodies in nucleated red cells (Fig. 10). Heinz bodies in erythroblasts were essentially similar in appearance to those seen in mature red cells and reticulocytes.

In addition to marked deposition of dense granules in the cordal macrophage and sinus lining cell, deposits were also present in the basement membranes of the sinus walls. This dense granular material, probably derived from degrada-

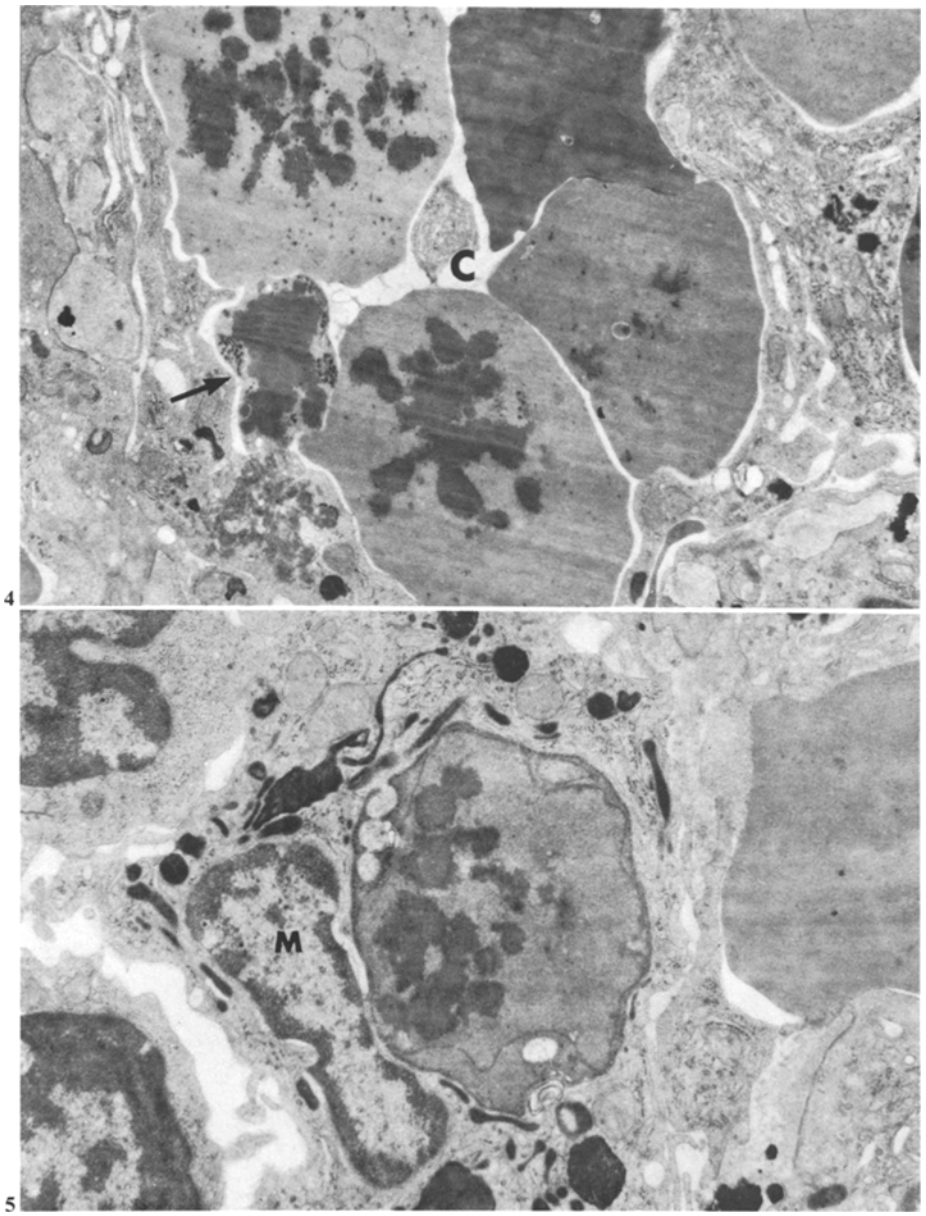
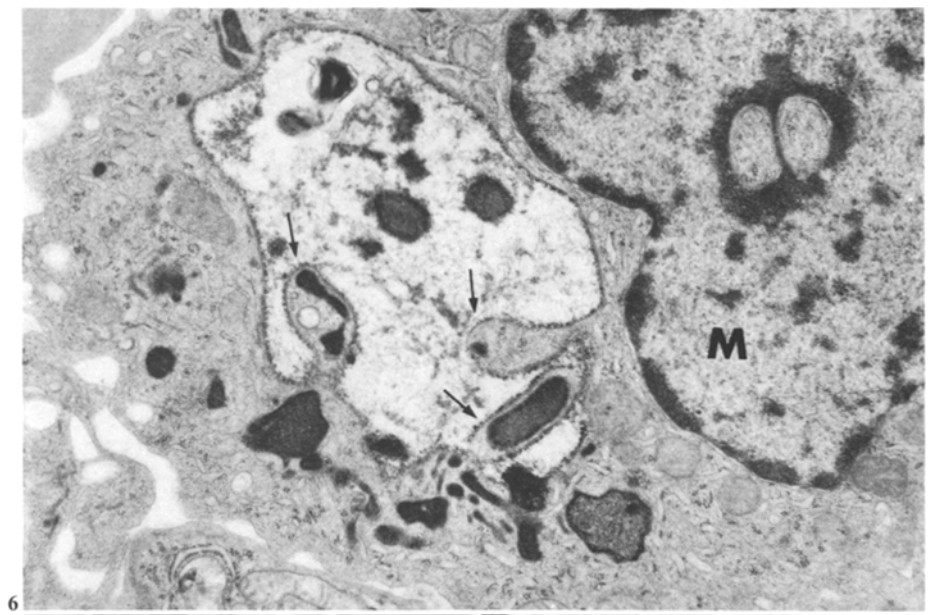


Fig. 4. The Billroth cord (*C*) is filled with Heinz body-containing red cells. Note a Heinz body fragment (arrow) in the cord. ×8600

Fig. 5. A Heinz body-containing erythrocyte recently engulfed whole by a cordal macrophage (*M*) reveals focal disruption of the cell membrane. The macrophage contains dense granular material. ×12,000



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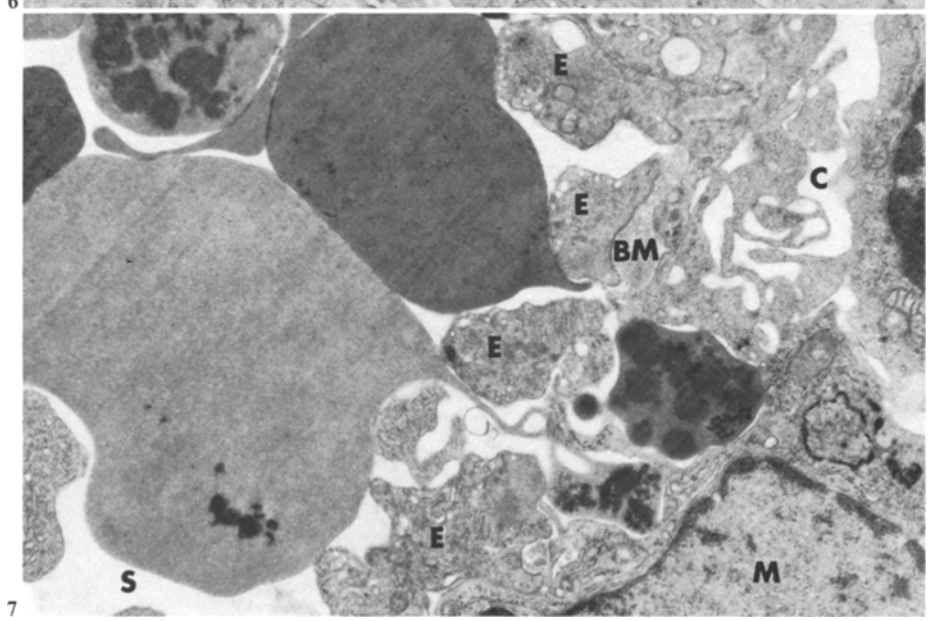

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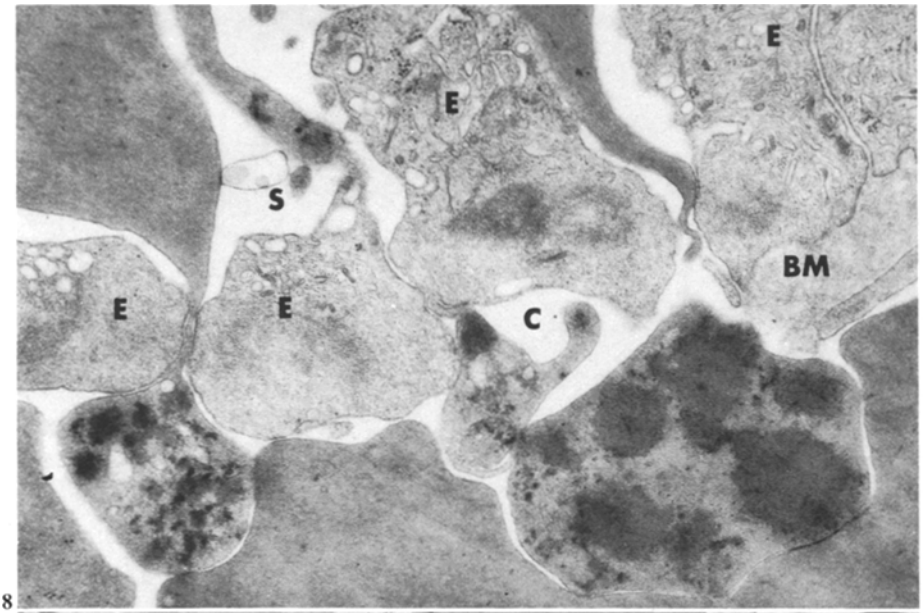
Fig. 6. A red cell engulfed by the macrophage (*M*) shows complete loss of hemoglobin and residual Heinz bodies. The erythrophagocytic vacuole appears to be divided into smaller phagocytic vacuoles by cytoplasmic protrusion (arrow). ×13,000

Fig. 7. Two Heinz body-containing red cells retained in the sinus wall. Heinz body-containing parts remaining in the cord (*C*) are encircled by cytoplasmic processes of the macrophage (*M*). *S* sinus; *E* sinus endothelial cell; *BM* basement membrane. ×11,000



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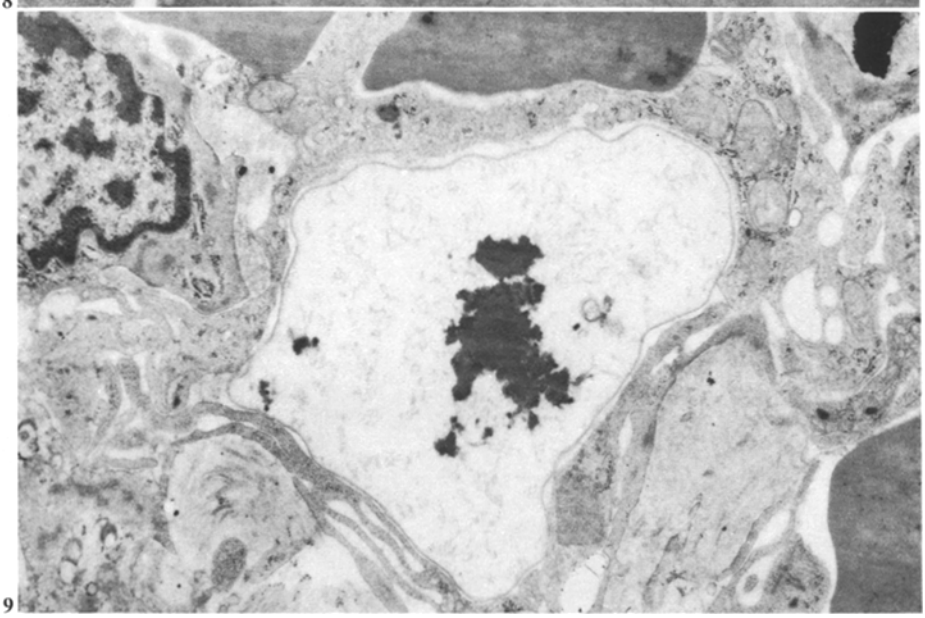

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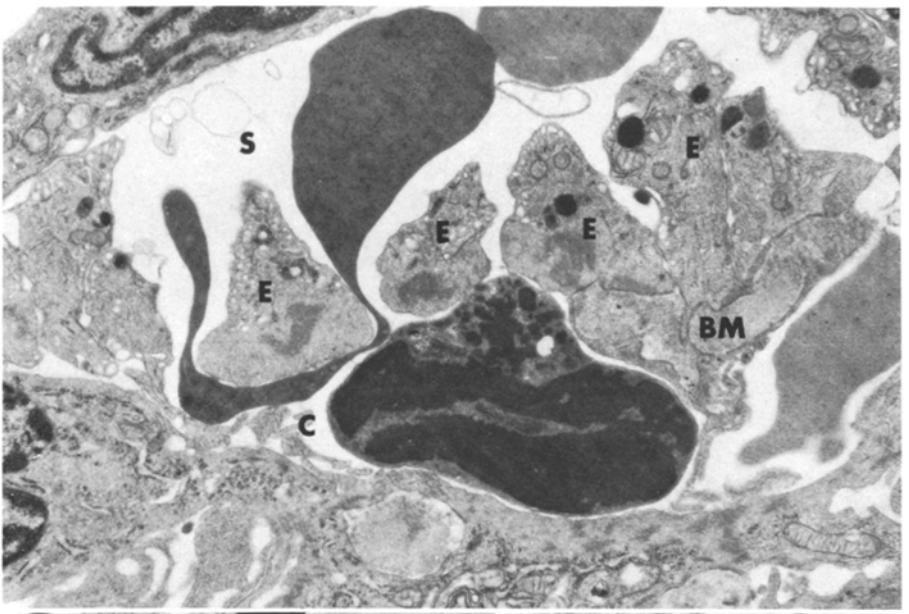
Fig. 8. Three Heinz body-containing red cells at the junction of the cord (*C*) and sinus (*S*). Breaks have occurred in the thin stalks that lie between the endothelial cells (*E*), leaving Heinz body fragments free in the cordal space. *BM* basement membrane. ×22,300

Fig. 9. A red cell ghost containing Heinz bodies is seen free within the cord indicating intravascular hemolysis in the splenic microvasculature. ×12,000



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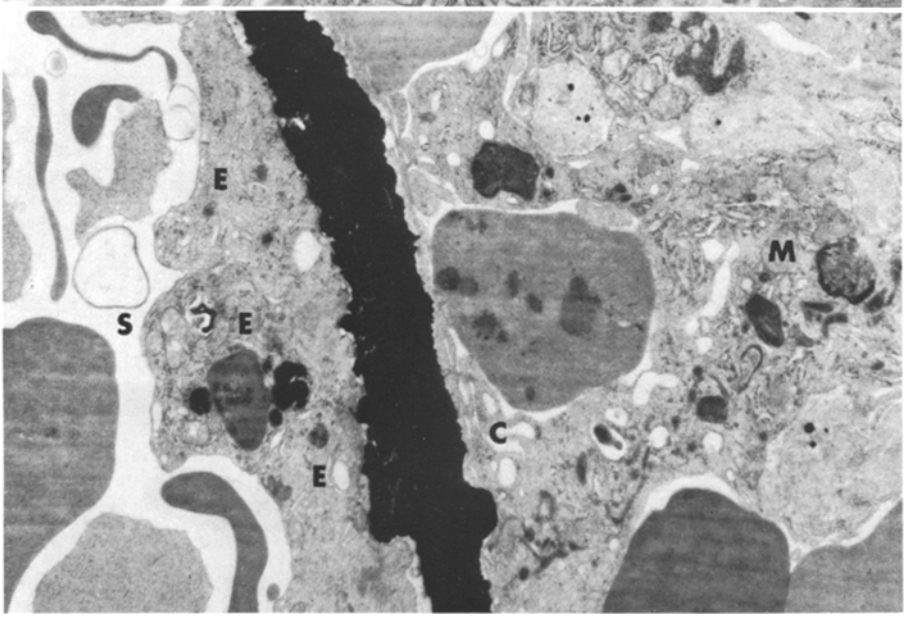

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Fig. 10. A nucleated red cell containing numerous Heinz bodies is seen in the cord (*C*) just beneath the endothelial cells (*E*). *S* sinus; *BM* basement membrane. ×9000

Fig. 11. Marked deposition of hemosiderin in the basement membrane of a sinus wall, which may be so stiffened as to have allowed the red cells to pass through its fenestration only with much difficulty. *S* sinus; *C* cord; *E* sinus endothelial cell; *M* macrophage. ×9000

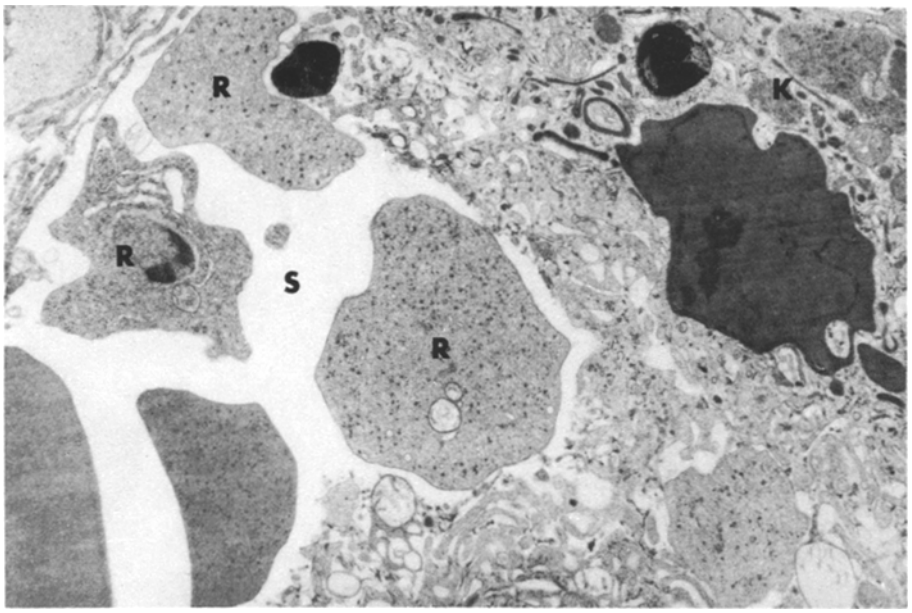

Fig. 12. Phagocytosis of a Heinz body-containing erythrocyte by a Kupffer cell (*K*). *R* reticulocyte; *S* sinusoid. ×8200

tion of Heinz body-containing red cells, was generally scattered within the basement membranes. In some areas, the basement membranes were completely filled with this material (Fig. 11).

Similar ultrastructural changes of the red pulp were noted in the accessory spleen. Phagocytosis of Heinz body-containing red cells in toto by macrophages and pitting of Heinz bodies were observed. Ghosts of hemolyzed red cells were occasionally found in the cords and sinuses.

Although erythrophagocytosis by Kupffer cells was occasionally found in the liver (Fig. 12), pitting of Heinz bodies by Kupffer cells was not demonstrated. There was no ultrastructural evidence of intravascular hemolysis within liver sinusoids.

Discussion

It is generally accepted that the spleen is capable of removing damaged red cells as a whole and has the ability to eliminate intracellular rigid bodies, such as iron granules (Crosby, 1957), malaria parasites (Schnitzer et al., 1972; Schnitzer et al., 1973), Heinz bodies (Rothberg et al., 1959; Koyama et al., 1964; Rifkind, 1965; Schnitzer and Smith, 1966; Lawson et al., 1969; Chen and Weiss, 1973; Leblond, 1973; Klausner et al., 1975), and dense inclusions in hemoglobin H disease (Wennberg and Weiss, 1968), from red cells without destroying these cells. The former mechanism has been termed a "culling"

function and the latter a "pitting" function of the spleen (Crosby, 1959). When red cells contain Heinz bodies, both culling and pitting are known to play an important role in removing Heinz bodies (Rifkind, 1965). Most of these observations were done in experimental Heinz body anemias in animals, for example dogs (Koyama et al., 1964), rabbits (Rothberg et al., 1959; Rifkind, 1965), rats (Schnitzer and Smith, 1966; Lawson et al., 1959; Chen and Weiss, 1973; Leblond, 1973), or mice (Klausner et al., 1975), following administration of oxidant drugs. To our knowledge, no literature concerning the fine structure of the red pulp of the spleen and the liver in unstable hemoglobin hemolytic anemia is available.

In this case, a marked improvement of hemolytic anemia and appearance of Heinz body-containing red cells in the peripheral blood following splenectomy suggested a significant role of the spleen in the removal of affected red cells. The principal mechanism of red cell destruction was phagocytosis of Heinz body-containing red cells by cordal macrophages. Erythrocytes with Heinz bodies are reported to have great difficulty in deforming their shape (Jensen and Lessin, 1970; Lubin and Desforges, 1972; Tillman et al., 1976). This is manifested morphologically by retention of red cells in the cord. Stagnation of these cells may facilitate prolonged and intimate contact of these cells with cordal macrophages finally resulting in erythrophagocytosis. The exact mechanism of how the macrophage recognizes these cells and the nature of the red cell membrane lesion that is recognized remain to be determined.

Some Heinz body-containing erythrocytes seem to be lysed in the cord without direct participation of macrophages. The presence of red cell ghosts free within the extracellular space of the cord supports this mechanism of hemolysis. There is some evidence to suggest that the erythrocytes containing Heinz bodies have an increased permeability of their membrane to cations (Jacob et al., 1968; Parker and Welt, 1972). In addition, it is also known that most Heinz bodies are attached to the cell membrane (Rifkind and Danon, 1965; Jensen and Lessin, 1970), although the precise mechanism of their attachment to the membrane is still in dispute (Jacob et al., 1968; Winterbourn and Carrell, 1973; Sears et al., 1975). Thus, it is likely that membrane alterations induced by the fixation of Heinz bodies to the cell membrane lead to intravascular lysis of such severely damaged cells in the splenic microvasculature. The degree of intravascular hemolysis of Heinz body-containing red cells in the general circulation could be negligible, since a minimal hemolysis was noted after splenectomy despite the persistent presence of such erythrocytes in the peripheral blood.

The erythrocytes which have escaped phagocytosis by cordal macrophages must traverse the interendothelial slits and narrow openings of the basement membrane. When red rells contain numerous Heinz bodies, they encounter great difficulty in passing through the sinus wall, especially the interendothelial slits recently emphasized by Chen and Weiss (1973). Heinz body-containing red cells retained in the sinus wall were lobulated into two parts; a larger part with or without tiny Heinz bodies lying in the sinus and the remaining portion containing the bulk of the Heinz bodies retained within the cord. As to the fate of these lobulated red rells, two processes of red cell fragmentation

were noted. One was partial phagocytosis of Heinz body-containing areas by cordal macrophages (Fig. 7) and the other was mechanical fragmentation within the interendothelial slits (Fig. 8). The latter mechanism of mechanical filtration of Heinz bodies in the sinus wall apertures has been shown in the spleen of mice (Klausner et al., 1975). Thus, pitting of Heinz bodies in the spleen can be achieved by either partial phagocytosis of Heinz body-containing cytoplasmic parts by the macrophage or mechanical fragmentation of the affected red cells at the sinus wall apertures.

Although the mechanisms of destruction of Heinz body-containing red cells in the spleen were essentially similar to those described in experimental Heinz body anemias in animals, a major difference was the presence of Heinz bodies even in nucleated red cells in this case, as opposed to their appearance exclusively in mature red cells and reticulocytes in the experimental models (Rifkind and Danon, 1965; Schnitzer and Smith, 1966; Lawson et al., 1969). This difference can be explained by the mechanism whereby these inclusions are formed. In experimental animals, administration of oxidant drugs results in the denaturation of natively intact hemoglobin in the red cells. As has been reported by Rifkind and Danon (1965), susceptibility of erythrocytes to denaturative effects of phenylhydrazine and related compounds may be proportional to cell age. Immature reticulocytes and nucleated red cells, for example, are more resistant to oxidative drugs by their enhanced capacity to reduce methemoglobin to hemoglobin. On the other hand, unstable hemoglobin, as evidenced by heat labile characteristics and increased precipitability by isopropanol, is so unstable as to be denatured spontaneously even in the presence of the increased reducing ability of the immature red cells. Similar instability of hemoglobins manifested by spontaneous development of inclusions in nucleated red cells has been reported in thalassemia (Fessas, 1963; Polliack and Rachmilewitz, 1973), and in patients with familiar hemolytic anemia associated with a defect of pigment metabolism (Schmid et al., 1959).

In the liver, phagocytosis of Heinz body-containing red cells by Kupffer cells was only occasionally encountered. Selective removal of Heinz bodies by Kupffer cells was not evident. These findings were consistent with observations of the liver in experimental Heinz body anemia in animals (Rifkind, 1965; Schnitzer and Smith, 1966). Red cell ghosts, a hallmark of intravascular hemolysis of injured red cells, were not observed in the liver. The liver sinusoids were considered to play a minimal role in the removal of Heinz body-containing red cells.

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Received February 18, 1977