Electron and Light Microscopic Study of the Erythroblasts of Patients With Congenital Dyserythropoietic Anemia

By Hermann Heimpe1, Jeronimo Forteza-Vila, Wolfgang Queisser and Egon Spiertz

The morphological changes of erythroblasts in congenital dyserythropoietic anemia type I have been analyzed by light and electron microscopy. The results support our earlier assumption, that this hereditary disorder of erythropoiesis is a disease entity and is not identical with any of the previously described hereditary hematological diseases. Characteristic morphological aberrations consist of widening of nuclear membrane pores, condensation, vacuolization and disintegration of the nuclear chromatin, structural changes of the nucleolus, appearance of myelin figures, and final autolysis of the cells. All changes are restricted to erythroblastic cells beyond the stage of the proerythroblast. The biochemical basis of this abnormality is not known. It leads to severe ineffective erythropoiesis with anemia, increased total hemoglobin turnover, and secondary hemochromatosis.

The term "congenital dyserythropoietic anemia" (CDA) has been applied by Crookston, Godwin, Wightman, Dacie, Davis, Lewis and Patterson1 and by ourselves2,3 to a group of hereditary refractory anemias, which are characterized by severe ineffective erythropoiesis, specific cytopathology of the nucleated red cells in the bone marrow and secondary hemochromatosis. Their pathogenesis is unknown. They are different from congenital sideroblastic anemia as described by Björkman4 and Heilmeyer, Keiderling, Bilger and Bernauer.5 There is no evidence of a hemoglobin anomaly or an alteration in vitamin B12 or folic acid metabolism. At least two disorders, tentatively classified as CDA type I and type II by Heimpel et al.,3 can be distinguished on the basis of the morphologic changes. This classification has been corroborated by the detection of specific membrane abnormalities of the mature red cells, which are present in all cases of CDA type II tested and not in type I.6,7

This report compares the results of light microscopic with electron microscopic studies of the bone marrow of four patients with CDA type I.

Clinical and Laboratory Findings in CDA Type I

CDA type I is a rare disorder of erythropoiesis of autosomal recessive mode of inheritance, as suggested by the occurrence in two pairs of sisters (case 1.
and 2, sister of case 3 of reference 3) and negative findings in parents and children as far as investigated. The disease is first manifested in infancy or adolescence. Moderate anemia and slight hyperbilirubinemia are the leading symptoms. Moderate splenomegaly is common. The anemia is macrocytic, but anisocytosis and poikilocytosis is prominent. Hemoglobin values range from 8.0 to 12.6 Gm. per cent, hematocrit from 25 to 36 per cent, RBC from 2.0 to \(3.3 \times 10^6/\mu l\) and MCV from 100 to 120 cu. \(\mu\). The number of WBC and platelets and the differential count are normal. Reticulocyte counts are slightly elevated and vary from 60,000 to 100,000/\(\mu l\). The hyperbilirubinemia is indirect with only trace amounts of direct bilirubin and total values of 1.4–3.5 mg. per cent. Varying degrees of direct hyperbilirubinemia were observed only in elder patients with hemochromatotic liver disease. Serum haptoglobin is lowered or absent. TIBC is low (220–280 \(\mu g\) per cent) and plasma iron is normal or elevated (130–280 \(\mu g\) per cent), the higher values being present in cases with more advanced secondary hemochromatosis.

The anemia is mainly due to ineffective erythropoiesis with additional peripheral hemolysis, as shown by the above-mentioned data, the severe erythroid hyperplasia, and the increased output of fecal urobilinogen. This concept is supported by the results of radioisotope studies: Plasma iron turnover is 5–10 times normal, but peripheral iron utilization is low (25–32\%). Red cell survival is only moderately shortened as measured by \(^{51}\)Cr (\(T_{50}\) 17–22 days). Surface counts after \(^{59}\)Fe revealed the typical delayed decrease of bone marrow activity present in ineffective erythropoiesis.

In our laboratory, investigations of red cell enzymes, hemoglobin electrophoresis and in vitro hemolysis tests have been consistently negative. Excretion of orotic acid has not been detected. Acidified serum tests have been carried out on all patients observed, using a minimum of 10 compatible sera. No positive results have been observed. The same is true for agglutination and lysis by anti-i. These results support the distinction from CDA type II, which shows also signs of ineffective erythropoiesis, but different morphological abnormalities of the erythroid cells.

Treatment with vitamin \(B_{12}\), folic acid, riboflavin, pyridoxin, and corticoids are worthless. Iron is contraindicated, because of the iron overload present in all cases. Splenectomy has been performed in some patients without benefit. There has been no need for regular transfusions in the patients studied. No progression of the anemia has been noted, but the condition of the patients may deteriorate in later life because of hemochromatotic changes of liver, heart, pancreas and endocrine glands.

**Materials and Methods**

*Light Microscopy*

Bone marrow aspirates were obtained from the posterior iliac crest. The marrow was aspirated into a syringe containing 0.5 mL EDTA solution (1% \(Na_2EDTA\) in 0.7% \(NaCl\)). Smears were made from the marrow spicules separated from blood on a sloping glass
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Plate. Panoptic stains were made according to the method of Pappenheim (fixation in 90% methylalcohol, 5 minutes; May-Grünwald solution, 5 minutes; Giemsa solution 10%, 20 minutes). For all solutions, distilled water buffered at pH 6.8 was used. For demonstration of iron, the prussian blue reaction was used. The nuclei were counterstained with hemalum solution. The PAS reaction was performed according to Pearse, applying pararosaniline for Schiff's reagent. The smears were also stained with Feulgen with a hydrolysis time of 12 minutes in 1 N HCl at 60°C. The modification of Graumann was used, applying pararosaniline for Schiff's reagent. Staining time was 45 minutes.

Electron Microscopy

The marrow spicules were immediately fixed in cold glutaraldehyde (3.5% in phosphate buffer pH 7.2) for 2 hours. The tissue was washed in the same buffer with Millonig solution for 1 hour at 4°C. Dehydration was carried out in ascending series of acetone, and the tissue was embedded in Araldite. The sections were mounted on copper grids coated with Formvar film and treated with uranyl acetate and lead citrate following the method of Venable and Coggeshall. Some of the grids were not treated by uranyl acetate and lead citrate to facilitate the differentiation of ferritin molecules and ribosomes, following the technique recently described by Bessis, Dreyfus, Breton-Gorius and Sultan. The sections were examined and photographed with a Siemens Elmiskop I-a using 80 kV.

Results

Light Microscopy

The E:G (erythropoietic:granulopoietic) ratios as assessed from enumeration of 1000 cells on the smears was between 2.1:1 and 4.7:1. An E:G ratio of 0.2–

Fig. 1.—Bone marrow smear, Pappenheim-stain. For explanation of symbols, see text.
0.8:1 is regarded as normal with the smear technique used. The majority of the red cell precursors beyond the stage of the proerythroblast and early basophilic erythroblasts show striking and typical morphological abnormalities. Different degrees of abnormal chromatin structure are seen in almost all of these erythroblasts (Fig. 1). In less severely affected cells, chromatin strands are of different thickness, but more gross than normal, unevenly distributed and interrupted by irregularly shaped translucent areas (Fig. 1,a). This ab-
normal structure is seen predominantly near the nuclear-cytoplasmatic border. In other cells, the chromatin structure has been partially lost and the nucleus shows large, homogeneous heavily staining and small translucent areas (Fig. 1,b). In the most severely affected erythroblasts the nucleus consists of an almost structureless, weakly stained material, which is not sharply delineated from the surrounding cytoplasm. The nuclear diameter varies from 6 to 20 μ and has no correlation to the structure of the nucleus or the degree of plasma hemoglobinization. Many damaged nuclei are seen even in technically optimal smears. There were only a few red cell precursors, which, according to the nuclear size and the homogenously condensed appearance of the nuclear chromatin, could be regarded as "oxyphilic" normoblasts.

Three forms of morphological aberrations, suggesting a disturbance of karyokinesis, are particularly typical: (1) Very large cells, which contain an

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Fig. 4.—Proerythroblast. The cells shows: nucleolus (Nu), nucleus (N), nuclear pore (np), cytosomes (cy), ribosomes (r), centriole (c), Golgi body (g), mitochondria (mi), ergatoplasmatic reticulum (er), and pattern of rhopheocytosis (→). × 18,000.
irregularly shaped nuclear mass consisting of two nuclear segments, suggesting incomplete nuclear division (1.1–2.0% of erythroblasts) (Fig. 2.c). (2) Double nucleated cells in which the two nuclei are of different size, chromatin structure and stainability. In some of these, the small nucleus appears as a budlike extension of the main nucleus (0.3–0.8% of all erythroblasts) (Fig. 2.d). (3) Pairs of erythroblasts connected by thin chromatin bridges of different length; see Fig. 3(1). Occasionally such bridges are seen between the two nuclei present in one cell. A positive staining with Feulgen ensures the presence of DNA in these connections (0.8–2.3% of all erythroblasts).

Approximately the same incidence of these abnormalities has been observed in all cases.

The percentage of erythroblasts containing iron-positive granules is moderately increased. Only occasionally "ring sideroblasts" with a continuous row of coarse granules surrounding the nucleus are seen. Less than 5 per cent of all erythroblasts contain small amounts of PAS-positive substances. No evidence of morphological abnormalities was present within the granulocytic and megakaryocytic lineages. Plasma cells and lymphocytes were seen in normal numbers. The percentage of macrophages containing large amounts of iron-positive material was somewhat increased. Many macrophages contained erythroblasts or erythroblastic nuclei.

**Electron Microscopy**

Like light microscopy, electron microscopy reveals unusual and significant morphological aberrations selectively within the erythroid series. Only pro-erythroblasts show normal ultrastructure of both nucleus and cytoplasm (Fig. 4). With progressing maturation, the pores of the nuclear envelope, which are a normal finding of erythroblastic nuclei, become more numerous and wider.
Fig. 6.—More severely affected cells. The cytoplasm (cy) penetrates between the chromatin (chr). The chromatin shows a spongy appearance. The nucleolus (Nu) is reduced to a few particles. × 12,000.

than normal (Fig. 5). Some nuclei have fingerlike protrusions surrounded by a small rim of cytoplasm. The nucleoli lack the filamentous component and
have a purely granular appearance. In later stages, corresponding to the late polychromatic normoblasts of light microscopic nomenclature, the dark chromatin becomes more dense than normal and forms sharply delineated, irregularly shaped clumps mainly at the periphery of the nucleus, containing small electron translucent vacuoles. In many cells the protoplasm has penetrated between the chromatin strands, as seen by the occurrence of cytoplasmic organelles between the dense chromatin (Fig. 6). In even more severely affected cells, the cytoplasm separates the chromatin remnants and gives to the nucleus a spongy appearance. The nucleolus is reduced to a thin, fragmented shell. Myelin figures appear in both cytoplasmic and nuclear areas (Fig. 7). In some cells, the cytoplasm is also vacuolated and contains large, pigmentlike inclusions and small floccular densities. A small fraction of erythroid cells are interconnected by a narrow sheath of cytoplasm containing dense chromatin, which merges into the marginal dense chromatin of both nuclei (Fig. 8). Other cells with abnormal chromatin structure present signs of incomplete division showing two nuclei attached to each other in a wide area (Fig. 9).

Masses of ferritin are seen in the cytoplasm of many red cell precursors. Siderosomes are more prominent than normal. Many mitochondria contain abundant deposits of ferrogenous micelles, causing a loss of normal structure in cristae and membrane (Fig. 10); these changes are particularly significant in reticulocytes.

The abnormalities described have been observed in all cases examined.

DISCUSSION

Comparative light and electron microscopic studies have been performed on the erythroblasts of four patients suffering from a recently described
Fig. 8 (Upper).—Bridge of dense chromatin sheathed by cytoplasm (→). × 50,000.

Fig. 9 (Lower).—“Normoblast.” Cell with abnormal chromatin (chr) shows incomplete division of nuclei. × 12,000.

hereditary disorder of erythropoiesis. It was initially named “congenital dyserythropoietic anemia”; in a subsequent publication, it was referred to as “congenital dyserythropoietic anemia type I” to distinguish it from a clinically similar, but morphologically different condition, which, independent from our observations, was termed CDA by Crookston et al.¹ The studies were undertaken with the aim to clarify the following questions:

(1) Are the cases described as CDA type I identical in their submicroscopic structure, and are they different from congenital anemias of known pathogenesis?

(2) How do the light microscopic aberrations correspond to the abnormalities in the electron microscope?
(3) How far do the morphological abnormalities help to elucidate the pathophysiological background of the ineffective erythropoiesis present?

The results were as follows:

(1) The striking morphological aberrations, as seen in panoptic stains with the light microscope, are qualitatively identical in all four cases. The same is true for the nuclear changes visible with the electron microscope. The quantitative morphological analysis was restricted to three types of aberrations that could be clearly defined and did not show a gradual transit to the normal state. Those aberrations were present in the bone marrow of the patients to approximately the same extent. The special appearance of condensed chromatin and the constellation and frequency of nuclear changes described, which were present in the vast majority of all erythroblasts except in the early proerythroblasts, has not been reported in any other clinical condition. Incidental features, however, such as rare chromatin bridges, budlike extensions and poor stainability of the nucleus can be observed in erythroleukemias, damage by radiation or drugs, the megaloblastic anemias or other refractory anemias. The abnormalities in the cytoplasm, especially the excess of iron in siderosomes and mitochondria, are also known from a variety of anemias. Myelin figures or "lamellar bodies" were described in the erythroblasts of patients with hemolytic anemia with an unstable hemoglobin.13,16

(2) The light microscopic changes in nuclear structure correspond to the intense clumpy condensation of the dense chromatin visible with the electron microscope. Erythroblasts, in which the different staining properties of nucleus and cytoplasm have been partially lost, are probably in a more advanced stage of destruction with cytoplasmic ground substance and organelles penetrating between the clumps of chromatin, as seen in Fig. 6. Giant nuclei with two segments (Fig. 2,c) consist of two nuclei attached to each other as seen in Fig. 9. They are probably the result of an incomplete nuclear division. One
of the most characteristic abnormalities, the consistent occurrence of chromatin bridges in bone marrow smears, has occasionally been observed in the sections for electron microscopy, revealing a string of dark nuclear chromatin connecting the nuclei of two erythroblasts and sheathed by a small rim of cytoplasm. Pairs of erythroblasts interconnected by chromatin bridges may also be the result of a disturbance of the chromatin division, identical but less severe than in cells of the type described above (Figs. 3 and 8).

(3) Visible morphological changes, in light as well as in electron microscopy, are restricted to the erythrocytic series. In addition, they are not recognized in the early basophilic stages, but start at the point where the increase of hemoglobin concentration becomes visible as polychromatophilia in panoptic stains. The different degrees of nuclear abnormalities, as seen in different cells of the polychromatotic stage, are probably consecutive steps of the same process of cellular deterioration leading to autolysis and final phagocytosis within the marrow. The first morphologically recognizable step in this sequence would then be a widening of the pores in the nuclear membrane, together with the disappearance of filamentous components of the nucleolus. If one assumes such a primary defect of the nuclear membrane, the condensation and vacuolization of the dense chromatin visible in the next stage of the pathological process could be secondary to the entrance of cytoplasmic material into the nucleus. There are a few erythroblasts in which widening of the pores is visible without change of the chromatin pattern itself, but this does not exclude the possibility, that a change of the dense chromatin, not visible at the beginning, is the cause rather than the result of the widened nuclear pores. The observation that the nuclear changes are absent in early basophilic stages and appear at the time of hemoglobinization favors the hypothesis of an interaction between normal hemoglobin and the nuclear membrane or material of the nucleus itself, possibly basic nucleoproteins as described by Tooze and Davies and Fawcett and Witebsky in nucleated amphibian erythrocytes.

Of particular interest are the myelin figures seen in the stage of distinct nuclear deterioration. Such myelin figures are generally accepted to be a late form of lysosomes. This opinion is supported by the observation of acid phosphatase in such multilamellated bodies in the nucleated red cells of amphibia. Because myelin figures are not detected in less severely affected cells, but appear rather late in the process of cellular decomposition, it is likely that lysosomal activation is a secondary event in the process of autolysis, in parallel to the effect of toxic compounds or ischemia on the lysosomes of liver cells. The increased amount of nonhemoglobin iron in the form of siderosomes and ferruginous micelles can be regarded as the expression of secondary hemochromatosis accompanying similar iron overload in other organs. The normal porphyrin values in urine and red cells speak against the primary importance of a sideroachrestic mechanism.

REFERENCES

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HERMANN HEIMPEL, JERONIMO FORTEZA-VILA, WOLFGANG QUEISSER and EGON SPIERTZ