Hereditary Dyserythropoiesis With Abnormal Membrane Folate Transport

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Dyserythropoiesis, which morphologically and serologically resembles congenital dyserythropoietic anemia type III but is not accompanied by anemia, is described in a young man. In addition to striking gigantism and multinuclearity of erythroid precursors, electron microscopy revealed widening of nuclear pores, nuclear clefts, and cytoplasmic inclusions. Membrane transport of 5-methyltetrahydrofolate by the patient's red cells was markedly reduced; total uptake, uptake velocity, and maximal velocity of uptake were all significantly less than in controls. In contrast, red cell uptake of pteroylglutamic acid was normal. Bone marrow cells in culture also showed decreased 5-methyltetrahydrofolate uptake, as well as very low thymidine incorporation. Because folate uptake by mitogen-stimulated lymphocytes was normal, the defect apparently does not involve all cell lines. These results suggest that a specific membrane defect, affecting the carrier system for reduced folate compounds, is present in this patient's erythrocytes, and perhaps, their bone marrow precursors.

The term dyserythropoiesis implies both quantitative and qualitative abnormalities of erythropoiesis. Dyserythropoiesis is characterized by anemia, morphological changes including asynchronous nuclear-cytoplasmic maturation, variation in nuclear size, structure and shape, multinuclearity, karyorrhexis, pyknosis, bridging, and mitotic abnormalities. Cytoplasmic abnormalities observed include the presence of vacuoles, basophilic stippling, increased cytoplasmic iron, and other inclusions. Dyserythropoiesis is caused by vitamin B₁₂, folate, or iron deficiencies, and improvement follows appropriate therapy. However, when dyserythropoiesis is associated with other hematologic conditions, such as sideroblastic anemia, myelofibrosis, or aplastic anemia, treatment with these hematinics is usually ineffective. Dyserythropoiesis also occurs as a group of congenital disorders. This rare and poorly defined group of diseases is further subclassified according to morphological changes in erythrocytes and erythroblasts and by serologic differences in the circulating red cells into congenital dyserythropoietic anemia (CDA) types I, II, and III. Nearly all of these cases have been associated with moderate to severe anemia, and most have been apparent during childhood.

This article describes a 30-yr-old white male with striking dyserythropoiesis without anemia. The patient was initially detected because of mild unconjugated hyperbilirubinemia discovered on a routine examination. The morphological and serologic findings in this patient most closely resemble CDA type III. In addition, his peripheral erythrocytes and bone marrow cells manifest abnormalities of

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Supported in part by grants from the National Leukemia Association, Inc., and by Grant CA 20251 from the National Institutes of Health. R.F.B. is a Special Fellow of the Leukemia Society of America, Inc.

Submitted May 8, 1978; accepted July 12, 1979.

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cellular folate metabolism. The peripheral blood of the 2-yr-old daughter manifested findings similar to the patient.

MATERIALS AND METHODS

Case History

The patient is a 30-yr-old office worker who was initially referred to University of Minnesota Hospitals because of persistent unexplained hyperbilirubinemia. Other than occasional sore throats in the previous 2 yr, he had been entirely well. He was known to have had a hemoglobin of 14.5 g/dl 5 yr prior to evaluation. In May of 1975, the hemoglobin was 13.5 g/dl. The patient had been treated with tetracycline for pharyngitis but received no other medications; he had no known exposures to heavy metals, insecticides, or other toxins. He denied jaundice or weight loss and did not know of any family history of anemia. His father and mother had both died of "heart attacks." He has three brothers, two of whom are normal and healthy; the third is mentally retarded. He is a conservative social drinker.

Physical examination was unremarkable; specifically, there was no jaundice, signs of lead poisoning or iron deficiency, and no hepatosplenomegaly or lymphadenopathy. The peripheral blood smear manifested a minor population of very macrocytic red blood cells and poikilocytes, including tear-drop forms and distorted red cells (Fig. 1). There was also fine and coarse basophilic stippling. The white count was 7 × 10^9/liter. The hemoglobin was 14.2 g/dl, hematocrit 40%, and red count 4.21 × 10^12/liter. The mean cell volume (MCV) was 94 cu μ, mean corpuscular hemoglobin (MCH) 34 μg, and mean corpuscular hemoglobin concentration (MCHC) 36%. The uncorrected erythrocyte sedimentation rate was 6 mm in 60 min and the reticulocyte count 2.2%. Acid and sugar water lysis tests were negative; as
was a serum hemolysis test using a battery of sera, including anti-I and anti-i. The serum bilirubin was
1.3 mg/dl, 0.2 mg of which was direct reacting. The lactic dehydrogenase (LDH) was 461 IU, (171 U
urea stable; normal range 27-67 IU). Serum heptoglobin was 17 mg/dl. His blood type was A Rh(−).
One brother was A Rh(+) and two A Rh(−). Serum iron was 137 mg/ml and iron-binding capacity
267 mg/ml with a saturation of 51%. The 51Cr red cell mass was 21 ml/kg; the T½ of labeled red blood
cells was 19 days with no evidence of splenic sequestration. Blood for lead and urine for other heavy
metals did not reveal evidence of abnormality. Leukocyte alkaline phosphatase was 91 U, (normal,
64-176 U). Cytogenetic analysis of bone marrow showed many cells with hyperdiploidy but no evidence
of chromosome structural abnormality. Serum folate was 11 ng/ml and red cell folate 338 ng/ml.
Urinary formiminoglutamic acid (FIGLU) excretion was 3.2 mg/24 hr (normal less than 3.0 mg/24
hr). Serum vitamin B12 was 410 pg/ml. Whole blood p50 O2 was 26.7 mm Hg. Tests for heat stability
and isopropyl alcohol stability of hemoglobin were normal. Starch gel electrophoresis revealed no
abnormal hemoglobin. Treatment with folic acid, 1 mg daily in divided doses for 4 mo, raised the serum
folic acid to 185 and red cell folate to 591 ng/ml, respectively, without affecting the hemoglobin
concentration, hematocrit, red blood cell indices, or peripheral blood morphology. The daughter's
peripheral blood also showed macrocytes, poikilocytes, and basophilic stippling (Fig. 1). Her hemoglobin
was 11.6 g/dl with normal red cell indices; white count was 8.3 x 109/liter with a normal differential,
and platelet count 243 x 109/liter. The serum folate was 21.5 ng/ml. The reticulocyte percentage was
1.7. Peripheral blood counts and morphology of the wife and three siblings were normal.

Morphological Studies

Wright-Giemsa-stained bone marrow and peripheral blood smears, as well as hematoxylin and eosin
stained sections of trephine bone marrow biopsies, were examined by light microscopy. Thin sections for
electron microscopy were prepared as previously described.5 Red cell ghosts were prepared and washed
by the method of Dodge6 and prepared for freeze fracture electron microscopy by the method of
Douglas.7 These same red cell ghosts were used for polyacrylamide gel electrophoresis as described by
Neville.8

Folate Uptake Studies

Peripheral blood was fractionated with a Ficoll-Hypaque gradient; lymphocytes were collected from
the interface and red cells were recovered from the bottom of the gradient. Cells were then prepared by
slight modifications of previously described methods.9 Briefly, lymphocytes were washed 3 times with
medium (“folate-free” Eagle’s medium, 10% dialyzed horse serum, 1% glutamine, 0.28% sodium
bicarbonate, and 1% penicillin-streptomycin) and adjusted to approximately 2 x 106 viable cells/ml.
Viability was assayed by trypan blue exclusion. One milliliter was placed in 12 x 75 mm tubes to which
phytohemagglutinin, 80 pg/mI (PHA-P, Difco Laboratories, Detroit, Mich.) was added. To some tubes,
6.3 µg 5-methyl-14C-tetrahydrofolic acid (5-14CH3-H4-folate; Amersham/Searle, Arlington Heights,
III.) was also added. After 72 hr of incubation at 37°C with 5% CO2, 0.1 µCi of 14C-thymidine was added
to appropriate tubes. All cells were then incubated an additional 17-20 hr, washed 3 times with 5 mM
phosphate-buffered saline (PBS), and collected by suction filtration onto Whatman CF/A filters. After
drying, the filters were covered with toluene-Liquiflor-Biosolv (New England Nuclear, Boston, Mass.)
and radioactivity measured.

Red cells were washed 3 times with PBS and resuspended to a hematocrit of approximately 40%.
Various concentrations of 5-14CH3-H4-folate were added in 0.1 ml of 20 mM ascorbate-saline to 1-ml
suspensions of erythrocytes. Initial samples (T0) were immediately washed 3 times with PBS and lysed
with 1 ml of water. Other samples were incubated for 45 min at 37°C, after which they were similarly
washed and lysed. A third group of samples (“recovery tubes”) was incubated without radioactivity; the
cells were then washed and lysed, and a known amount of radioactivity added to the lysate. Red blood
cell counts were performed before and after incubation and washing to assure that no loss of cells
occurred. Samples were also inspected visually for hemolysis. Thereafter, all samples were autoclaved,
centrifuged at 800 g, and radioactivity in 0.5 ml of supernatant measured. Total intracellular uptake was
computed by subtracting T0 values from incubated values and multiplying this result by the ratio of
radioactivity added to “recovery tubes”: supernatant radioactivity in “recovery tubes.” All determina-
tions were done in triplicate. Because of the large amount of blood required, similar but more limited
studies were performed on red blood cells of the daughter and two age-matched controls. Measurements
of 5-14CH3-H4-folate uptake by bone marrow cells of the propositus were performed as described.10 All
determinations were done in triplicate.
RESULTS

Morphology

Bone marrow examination revealed normal thrombopoiesis and myelopoiesis. There was erythroid hyperplasia with prominent multinuclearity, gigantism, open nuclear chromatin pattern, karyorrhexis, and karyolysis (Fig. 1). In Prussian-blue
treated smears, many of the erythroid precursors, both uninucleated and multinucleated, contained multiple particles of iron. In some instances, the particles of iron encircled the nucleus or nuclei. The erythroblasts were uniformly periodic-acid-Schiff negative. Ultrastructural studies showed multinuclearity with some of the nuclei in an individual cell appearing to be of different stages of maturity (Fig. 2). There was widening of nuclear pores, nuclear clefting, and dense cytoplasmic inclusions. Some of the nuclei had a spongy appearance. There was no evidence of nuclear or cytoplasmic bridging, cytoplasmic organelles within the nuclear envelope, or a double cytoplasmic membrane. Gaucher-like cells were not seen.
Freeze Fracture

Freeze fracture of red cell ghosts obtained from the patient showed randomly distributed intramembrane particles indistinguishable from control ghost preparations (Fig. 3).

Acrylamide Gel Electrophoresis

Acrylamide gel electrophoresis of red cell ghosts showed a banding pattern that was indistinguishable from a simultaneously tested sample from a normal individual.
Fig. 3. Freeze fracture of red cell ghosts of the patient (left) and a simultaneous normal control (right) (×120,000). Intramembrane particles are randomly distributed, the patient being indistinguishable from the control ghosts.

Folate Studies

Uptake of 5-[^14]CH_3-H_4-folate by patient red cells was markedly decreased compared to uptakes by erythrocytes from 12 normal individuals (Fig. 4). On three separate occasions, patient uptakes after 45-min incubation were below two standard deviations of the mean of control uptakes at all concentrations tested. A double reciprocal plot of these data produced a $K_m$ of 0.27 μM and a $V_{max}$ of 0.095 pmole/10⁹ cells/min for the patient’s red cells. Similar analysis of the data from normal controls showed a $K_m$ of 0.50 ± 0.12 μM and a $V_{max}$ of 0.301 ± 0.082 pmole/10⁹ cells/min. Analysis of a limited single study of the daughter showed a high $K_m$ of 0.93 μM and a $V_{max}$ of 0.46 pmole/10⁹ cells/min. Similar studies in two age-matched controls showed $K_m$s of 0.68 and 0.19 μM with $V_{max}$ of 0.45 and 0.56 pmol/10⁹ cells/min. The wife was also studied on one occasion. Her $K_m$ and $V_{max}$ were within the normal range ($K_m$, 0.44 μM; $V_{max}$, 0.232 pmole/10⁹ cells/min).

5-[^14]CH_3-H_4-folate uptakes by red cells from the patient’s 3 hematologically normal brothers were at the lower limits of the normal range (Fig. 4). Derived kinetic parameters were: $K_m$, 0.42, 0.25, and 0.34 μM, and $V_{max}$, 0.146, 0.106, and 0.141 pmole/10⁹ cells/min, respectively.

Incubation of erythrocytes from 13 normal individuals with 50 ng of ³H-pteroylglutamic acid (³H-PGA) for 45 min at 37°C resulted in an uptake of 35.3 ±
11.1 pg/10⁹ cells. ³H-PGA uptake by the patient's erythrocytes was 24 pg/10⁹ cells.

Following a 4-hr incubation of patient bone marrow cells with 6.3 μg 5-¹⁴CH₃-H₄-folate, uptake as 1.11 ng/10⁶ cells. This value is substantially lower than uptakes we previously reported for cells from nonanemic individuals¹⁰ or by cells from 4 patients with refractory macrocytic anemia (mean 2.91, range 1.29–3.59 ng/10⁶ cells). More striking, however, was the very low level of ¹⁴C-thymidine incorporation by bone marrow cells from the patient in parallel cultures. Only 30 cpm/10⁶ cells were measured, compared to a mean of 710 (range 270–1250 cpm/10⁶ cells) in 7 nonanemic marrows, and a mean of 460 (range 180–850 cpm/10⁶ cells) in bone marrow cultures from 4 patients with refractory anemia.

Uptakes of 5-¹⁴CH₃-H₄-folate by mitogen-stimulated lymphocytes from the patient on 2 occasions were 3.52 and 2.65 ng/10⁶ cells. These values were within the 95% confidence limits for determinations in 7 normal individuals (2.18–6.62 ng/10⁶ cells). ¹⁴C-thymidine incorporations by his stimulated lymphocytes (765
and 1268 cpm/10^6 cells) were similarly within the normal range (708–1532 cpm/10^6 cells).

**DISCUSSION**

Most congenital cases of dyserythropoiesis have been subclassified into CDA types I, II, or III, based on morphological and serologic studies, although other variants have been described. In general, the morphological features of the present case most closely resemble CDA type III. Macrocytosis was present, but the marked abnormalities of peripheral blood erythrocytes typical of types I and II were absent, as were nuclear and cytoplasmic bridging and intranuclear inclusions in red cell precursors. Although some binucleate forms were seen in the bone marrow, the most prominent findings were multinucleate forms and gigantoblasts, which are characteristically found in CDA type III. The cisternae of smooth-surfaced endoplasmic reticulum and Gaucher-like cells characteristic of CDA type II were not found. Disruptions of the nuclear envelope, as seen in CDA I, were observed. Although sideroblasts have been demonstrated in CDA III, the ring sideroblasts observed in this case are not a consistent feature.

Serologic characteristics, as well as morphological features, have been utilized to distinguish the various types of CDA. The present case demonstrated negative acid and sucrose hemolysis tests on repeated occasions, and although there was moderate lysis with anti-I and anti-i, it did not exceed control red cell lysis. These serologic findings differ somewhat from those previously described for CDA type III, wherein moderate lysis occurred with anti-I and anti-i. This difference, plus the absence of anemia, suggests that this patient’s disorder may not be typical CDA type III. However, the affection of the daughter suggests a hereditary cause, possible autosomal dominant inheritance. Previously reported cases of CDA type III have followed this genetic pattern, although a family history is not invariably found.

Because dyserythropoiesis occasionally develops in patients with vitamin B12 or folate deficiency, these possibilities were investigated in our patient. Measurement of serum and red cell folate and serum B12 levels were all within the normal range in the propositus, and the daughter had an elevated serum folate level. These observations argue against simple vitamin deficiency as a cause of the erythropoietic defect. Nevertheless, when more detailed studies of cellular folate metabolism were performed, abnormalities were found in 5-^14^CH3-H4-folate uptake by circulating erythrocytes and bone marrow cells of the propositus. On 3 occasions, total uptake, velocity of uptake, and maximum transport velocity (Vmax) for 5-^14^CH3-H4-folate by red cells were all significantly decreased. Further, the observed reduction of 5-^14^CH3-H4-folate uptake by this patient’s bone marrow cells is consistent with the possibility that a similar abnormality is present in precursor cells. However, the bone marrow results must be considered tentative, since we could not correct for the known abnormalities of nucleic acid synthesis in these patients. It would have been desireable to relate net folate transport in bone marrow precursor cells to nucleic acid synthesis. However, since many erythroblasts were multinuclear, and nuclei varied in maturation within a given cell, the methodology was not available for this correlation. The transport defect did not occur in all cell lines because uptake of 5-^14^CH3-H4-folate by mitogen-stimulated lymphocytes was
normal. In addition, transport of the oxidized form of the vitamin, pteroylglutamic acid, by red cells was normal. These results suggest that a rather specific membrane defect, affecting the carrier system for reduced folate compounds, is present in this patient's erythrocytes. Only limited studies could be performed on the daughter because of difficulty obtaining adequate blood samples from her and age-matched controls. Nevertheless, we found an elevated $K_m$, suggesting a reduced affinity of the transport system for 5-$^{14}$CH$_3$-H$_4$-folate.

Red cell membrane defects have been reported in other types of CDA, particularly type II (HEMPAS). Binding studies of anti-I, anti-i, and diverse antisera strongly suggest that CDA II cells manifest altered surface characteristics. Moreover, these cells have a decreased negative charge with reduced surface neuraminic acid levels. Changes have also been noted in the lipid composition of CDA II red cell membranes and in membrane proteins. Studies of anti-I and anti-i hemolysis in our patient and of erythrocyte membrane proteins by polyacrylamide gel electrophoresis were normal. Moreover, membrane morphology was indistinguishable from normal by electron microscopy of freeze fracture replicas. Nevertheless, our evidence of decreased folate transport suggests the presence of a membrane defect. Whether this impairment of membrane function contributes to this patient's dyserythropoiesis or is secondary to another cellular metabolic abnormality is presently unknown. Folate membrane transport and intracellular utilization are intimately linked. Thus, in situations where cellular folate metabolism is impaired, as in pernicious anemia, folate uptake is reduced. Since folate is involved in nucleic acid synthesis in the formation of thymidylate and others have found evidence of abnormal nucleic acid metabolism in CDA, the possibility that a defect in nucleic acid synthesis resulted in the reduction of folate uptake must be considered. Alternatively, the observed abnormalities of folate transport and the morphological changes of red blood cells and precursors may be entirely unrelated, since the three brothers exhibited abnormal folate transport but had entirely normal red cell morphology. Although this patient's disease most resembles CDA type III, distinctive features were noted. Therefore, we do not know whether the folate transport abnormality is unique to him or may be present in other patients with CDA. Further studies will be necessary to determine if defective cellular folate metabolism accompanies the disordered red cell maturation in these patients.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Jan Parkin, Jane Swanson, Bruce Anthony, Holli Stotesbery, and Marj Tuhy.

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