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Erythrokinetics in Cooley's Anemia

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COOLEY'S ANEMIA was established as a disease entity by Cooley and Lee in 1925.[1] Its characteristic alterations in red cell morphology of microcytosis and hypochromia resemble iron deficiency.[2] However, body iron stores are usually increased,[3, 4] and if any relation exists between these two disorders, it has been assumed to relate to impairment in hemoglobin synthesis.[5] Rich[6] is of the opinion that the Cooley gene abnormality blocks the production of normal hemoglobin and causes the continued production of fetal hemoglobin. Crosby and Akeroyd[7] have presented calculations indicating a failure to produce red cells at a rate comparable to that observed in some other hemolytic anemias. The concept of a relative impairment in marrow function has been voiced by others.[8-10]

Red cell survival studies[11-13] indicate that accelerated destruction of circulating red cells is at least a contributory cause of anemia. The absence of any demonstrable extracorpuscular component,[12] except as an acquired complication secondary to splenomegaly,[14] and the proven hereditary nature of the disease,[15] have resulted in the classification of Cooley's anemia as a genetically determined hemolytic anemia resulting from an intracorpuscular defect.

Combined studies of erythropoietic activity, of red cell destruction and rates of iron metabolism have been undertaken by us to gain further insight into the nature of Cooley's anemia. Although the results support the concept of an increased rate of turnover of the circulating erythrocyte mass, these studies revealed the major abnormality determining the degree of anemia is in red cell production.

MATERIALS AND METHODS

A. *Patients investigated*

Four patients with thalassemia major were studied. The first two, Ch. L. and Co. L., had an intermediate degree of anemia. In a previous report detailed clinical, hematologic and genetic data including special biochemical and electrophoretic studies were presented.[16] In brief, patient 1, Ch. L., at the time of this study, was 9 years old and his sister, patient 2, Co. L., was 13 years old. For most of their lives, both were known to have anemia. However, blood transfusions were not required to maintain their hemoglobin at concentrations compatible with a fairly normal existence. Both parents are of Italian ancestry and have typical microcytic erythrocytes with hemoglobin and serum iron concentrations within normal limits. Splenectomy had been performed on Co. L. approximately 2 years prior to the present study. Except for persistent splenomegaly and mild pallor, the patients' physical examinations had been within normal limits. Examination of their blood at this time revealed a

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microcytic anemia of 7 to 8 grams hemoglobin. In tables 1 and 2 are found results of other routine blood tests performed at the time of this study.

The other two, patients 3 (M. La) and 4 (P. M.), 4½ and 6½ years old respectively, have been classified clinically as examples of severe Cooley's anemia because blood transfusions were required to maintain their hemoglobin at minimal concentrations permitting moderate physical activity. The parents of both of these patients are of Italian ancestry and have the typical microcytic erythrocytes without anemia characteristic of Cooley's trait. Ten months prior to the present series of investigations, splenectomy had been performed on patient 4 and both patients had received blood transfusions approximately 2 months previously. The pertinent hematological data of these four patients are recorded in table 1.

B. Methods

Marrow differential counts were performed on 4000 cells (only myelocytic and erythrocytic cells were counted). Reticulocyte counts were performed on dried preparations previously stained with cresyl blue, and 4000 red cells were counted. Urobilinogen was measured according to the method of Watson et al.[17] with slight modification.[18] Cr^{51} survival was performed according to the method described in detail by us.[19] Care was taken that the concentration of chromium did not exceed 25 γ per ml. of red cells, since greater concentrations of the chromium iron have been shown to be toxic to the normal cell. Activity was expressed as cpm/ml. of whole blood, and survival was calculated as previously described with allowance for elution. The plasma volume was determined by extrapolating the Fe^{59} plasma activity to 0 time and dividing this figure into the counts injected. Total blood volume and cell volume was calculated from the plasma volume, employing a correction of 0.9 in the venous hematocrit.[20] This agreed within ± 10 per cent of calculations of blood volume based on body weight.

The plasma iron turnover and red cell utilization were measured according to the method of Huff and associates.[21] Approximately ½ μc of Fe^{59} per kilo body weight, as ferrous citrate, was incubated for half an hour with freshly obtained normal plasma containing several times the iron binding capacity required. Following intravenous injection of a weighed amount of this tagged plasma, samples were taken during the following four hours to determine the clearance rate of radioiron from the plasma. Samples were thereafter obtained every twelve hours for the next four days to measure the incorporation of radioiron into the red cells and then less frequently over the following two weeks. Plasma iron was determined by the method of Kitzes and Elvejem.[22] Plasma iron turnover was calculated according to the following formula.[21]

$$\text{Plasma Iron Turnover (mg./day)} = \frac{0.693 \times \text{Plasma Iron } (\gamma) \times \text{Plasma Volume (ml.)} \times 1440}{\text{T } ½\text{(min.)}}$$

Red cell utilization was determined by dividing the maximum counts of radioactivity found in circulation during the first 14 days by the counts injected. The amount of iron incorporated daily in erythrocytes was calculated as follows:

$$\text{Iron for erythropoiesis (mg/day)} = \text{Plasma iron turnover (mg/d)} \times \% \text{ util.}$$

To measure red cell production in these patients, it was necessary to express the data according to certain standards which permit comparison between individuals of different size. It was presumed that the measurements applied equally to children and adults since their red cell turnover occurs at similar rates.[23] The expressions employed and the average data previously obtained in normal adults[18] are as follows:

Measurements	Normal	Expression
Erythroid marrow	463.	nucleated rbc/1000 myelocytic cells
Reticulocyte	1.28	retics/1000 rbc
Plasma Iron Turnover	61.	γ/Gm. hemoglobin/day
Red Cell Utilization of Radioiron	46.	γ/Gm. hemoglobin/day
Urobilinogen Excretion	0.22	mg/Gm. hemoglobin/d

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To reflect marrow activity quantitatively, the measurement obtained was *compared to the expected measurement in that individual were he to have a normal red cell mass and normal blood production.* It was thus necessary in the anemic subject to redefine the above expressions, so that the erythropoietic or production figure related to the *normal* red cell mass, red count, or hemoglobin of the individual. Specific formulas which have been discussed elsewhere in more detail[18] are as follows:

	Production
Erythroid marrow	nucleated rbc/1000 myeloid cells
Reticulocyte	$\text{retics/1000 rbc} \times \frac{\text{pt. rbc/cu. mm.}}{5 \times 10^6}$
Plasma Iron Turnover	$\frac{\text{PIT } (\gamma/\text{d})}{\text{patient's normal circulating Hb. (Gm.)}}$
Red Cell Utilization	$\frac{\text{PIT} \times \%\text{ utilization } (\gamma/\text{d})}{\text{normal circulating hemoglobin (Gm.)}}$
Urobilinogen	$\frac{\text{Urobilinogen (mg./d)}}{\text{normal circulating hemoglobin (Gm.)}}$
Cr^{51} survival	$\frac{120}{\text{pt. red cell survival}} \times \frac{\text{pt. rbc/cu. mm.}}{5 \times 10^6}$

By dividing the patient's value of *production* for any one measurement by the average normal value listed above, a *production index* was obtained.

In handling data of those patients with Cooley's anemia, because of the marked distortion in size and hemoglobin concentration of their erythrocytes a further series of calculations were made. The marrow, reticulocyte and Cr^{51} production indices were multiplied by pt.'s mean corpuscular hemoglobin/normal mean corpuscular hemoglobin. This, in effect, changed all indices so they were proportionate to the hemoglobin mass, rather than cell unit.

Results

General hematologic data summarized in table 1 indicates that two of these children (Ch. L. and Co. L.) had a mild anemia and two (M. La. and P. M.) were more severely anemic. In two there was a reduction in average cell size below normal, and the mean corpuscular hemoglobin concentration was decreased in all. Nucleated erythrocytes and shape abnormalities were most con-

Table 1.—*Erythrokinetics*

Patient	I Ch. L	II Co. L.	III M. La.	IV P. M.
Blood volume (ml.)	2250	2530	1150	1840
Plasma Volume (ml.)	1670	1780	960	1470
Hematocrit (%) (venous)	28	32	18	22
Hemoglobin (Gm. %)	7.8	8.0	4.7	5.1
MCV (μ^3)	58	72	86	80
MCHC (%)	28	25	26	23
Nucleated Red Cells/1000 r.b.c.	0.1	11	2.7	18
Serum Iron (γ%)	62	167	208	183
Previous Transfusion	0	0	yes	yes
Splenectomy	no	yes	no	yes

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spicuous in the two patients who had been splenectomized. Serum iron concentration was depressed in one patient (Ch. L.) and elevated in the others.

Special studies are summarized in table 2. There was marked relative erythroid hyperplasia of the marrow with a somewhat greater increase in those patients most seriously affected. Reticulocyte counts were elevated to 10 and 15 per cent in the patients with mild anemia, and in the two with severe anemia were 2.4 and 3.6 per cent. Plasma iron turnover was greatly increased, but the per cent of injected radioactivity appearing in the red cell mass was reduced below normal in all. The mildly anemic children had 20 to 30 per cent utilization as compared to 4 and 10 per cent in the severely anemic patients. The shapes of the utilization curves were of particular interest in that only one of the four patients showed evidence of cell destruction during the first few days (fig. 1). Fecal urobilinogen was increased to between 400 and 600 mg./day, as compared to anticipated values of 40–60 mg./day for normal individuals with red cell mass equivalent to these patients' predicted normal mass. Cr^{51} survival was significantly shortened. In contrast to a normal half-time disappearance of radioactivity (T ½) of 26 days, these patients had half-times of 10, 7 and 9 days.

These erythrokinetic data have been expressed according to the formulas presented under Methods. The use of methods, some of which relate to red cell destruction in calculating erythropoiesis, is only valid if a state of equilibrium exists. This was true in the patients with mild anemia, and the slight fall observed in the patients with mild anemia, and the slight fall observed in the severely anemic patients during study was thought not to affect the results seriously.

In table 3, it will be seen that production indices for the first two patients with mild anemia in general were about 8 times normal (5 to 11 times normal) except for red cell utilization which was 2–3 times normal. Production indices, for the two more severly anemic patients, derived from marrow erythroid/myeloid ratio, plasma iron turnover and urobilinogen averaged 8 times normal (6 to 12 times normal). However, indices derived from reticulocytes, red cell utilization and Cr^{51} survival more closely approximated normal.

Some of these indices referred to red cell production and others to hemoglobin production. Since the erythrocytes of these patients did not contain a normal amount of hemoglobin, these indices were not strictly comparable. In table 4, *hemoglobin production indices* are compared, and it is seen that intramedullary production of hemoglobin which is measured by erythroid marrow, plasma iron turnover and urobilinogen is roughly similar in all patients (approximately 6 times normal). However, measurements expressing production of circulating

TABLE 2.—*Erythrokinetic Data*

Patient	Marrow (normoblasts/ 1000 myelocytic cells)	Reticulytes %	Serum Iron Turnover mg./d	Red Cell Utilization		Urobilinogen mg./d	Cr^{51} Survival (T ½ in days)
				%	mg/d		
Ch. L.	3731	10.2	120	19	23	436	10
C. L.	3902	15.6	124	31	38	635	7
M. La	5711	2.4	50	3.3	1.6	—	—
P. M.	4714	3.6	90	11	10	518	9

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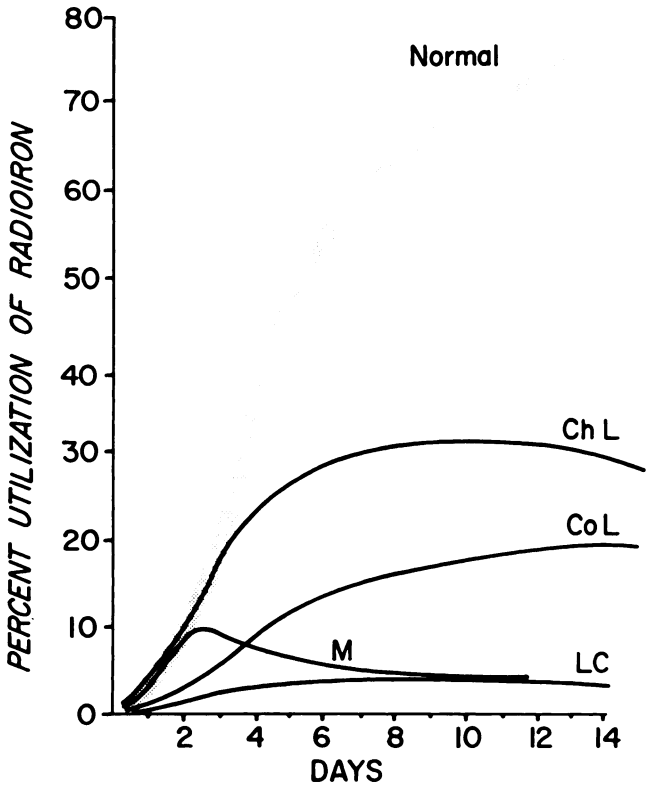

FIG. 1.—The appearance of radioiron in the circulating red cell mass of patients with Cooley's anemia is compared with normal subjects (shaded line). It will be observed that there is a marked reduction in red cell radioiron in the two severely anemia patients (M., LC.) and the reduction to one-third normal in the two mildly anemic patients (Ch. L., Co. L.).

TABLE 3.—*Erythropoietic Indices*

Patient	Marrow	Reticulocytes	Plasma Iron Turnover	Red Cell Utilization	Urobilinogen	Cr^{51} Survival
Ch. L.	8	7	6	2	7	6
Co. L.	8	11	5	3	9	9
M. La.	12	1.0	6	0.2	-	—
P. M.	10	2.0	7	1	10	4

TABLE 4.—*Hemoglobin Production Indices*

Patient	Marrow	Plasma Iron Turnover	Urobilinogen	Reticulocyte	Red Cell Utilization	Cr^{51} Survival
Ch. L.	5	6	6	4	2	3
Co. L.	5	5	8	6	3	3
M. La.	8	6	—	1	0.2	—
P. M.	6	7	8	1	1	2

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hemoglobin, i.e., reticulocyte count, red cell utilization of radioiron and Cr^{51} survival, are less: approximately 4 times normal for the mildly anemic patients and in the severely anemic patients not increased above the production rates of non-anemic normal subjects.

Discussion

Red cell destruction in Cooley's anemia as measured by the Ashby technic[11-13] has been reported to occur at a rate from 2 to 5 times normal. The half-time disappearance of Cr^{51} tagged red cells of 10, 9 and 7 days reported here if converted to destruction rate by appropriate formulae[19] indicate a rate of 7 to 10 times normal. This greater destruction rate could reflect the true destruction rate of the patient's own cells within himself, or may be erroneously high as the result of either excessive elution or damage to the erythrocyte by the chromium ion. It is of interest, however, that the rate of destruction in any case is insufficient to produce severe anemia, if the compensatory increase in erythropoiesis usually found in other hemolytic anemias occurred here.[18] It was of further interest that comparable rates of destruction were found in the two moderately anemic patients and the one severely anemic.

While further data are needed to delineate the degree of hemolysis in these cases and to distinguish between primary effects of the disease versus the secondary effect of splenomegaly,[25] these limited studies emphasize the abnormality in erythropoiesis as the major factor contributing to the anemia in these patients.

The quantitation of blood production is beset with difficulties. Any single method is open to certain criticism[18] and figures obtained must be regarded as approximate. By employing a number of measurements, some perspective is obtained on the consistency of individual determinations and some generalizations concerning the behavior of the erythron may be made. Blood production, as measured by the erythroid marrow, serum iron turnover and urobilinogen excretion, was increased in all patients to maximal values. Previous studies have indicated that production in the adult, as measured by these methods, may increase to 6 times normal.[18] These children appeared to have increased to about 8 times normal, but when the indices were recalculated on the basis of hemoglobin production (table 4), production was approximately 6 times normal. It was of particular significance that the hemoglobin production by these three measurements was of equal magnitude in all 4 subjects. In a previous paper, it was indicated that the relative number of nucleated erythrocytes, the iron assimilated by the marrow and the pyrolle pigment excreted were functions of *total hemoglobin production*. They did not distinguish between a turnover of heme constituents within the marrow and the actual delivery of red cells to the peripheral blood.

The remaining 3 measurements (reticulocyte count, red cell utilization of of radioiron and Cr^{51} survival) related to the circulating red cell mass and thus measured cells delivered from marrow to circulating blood, i.e., *effective red cell production*. Here there was a considerable difference between the mildy anemic patients with an effective red cell production rate of about 4 times normal and the two severely anemic patients who were unable to increase effective red cell production above that of the normal non-anemic subject.

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Thus, the mildly anemic and severely anemic patient with Cooley's anemia appeared to have comparable rates of destruction of circulating erythrocytes and of total hemoglobin production. The difference between the two was in the percentage of effective red cell production to total hemoglobin production. In the mildly anemic patient, this ratio was approximately 3 to 6, or 50 per cent effective, while in the severely anemic patient it was 1 to 6, or 15 per cent effective. Such ineffective erythropoiesis has been described in pernicious anemia[24] and suggested in refractory anemia.[18] Actually, these measurements permit only the conclusion that the increase in erythroid marrow and the iron turnover and pyrolle pigment excretion occurred in excess of the amount required by the circulating hemoglobin mass, allowing for its accelerated rate of destruction.

As indicated in figure 2, all gradations in this process may be anticipated from the patient who shows primarily the picture of a compensated anemia, to the severely anemic patient who showed little evidence of blood production and might be classified as an aplastic anemia (with hyperplastic marrow). This is consistent with the wide range of severity observed clinically in Cooley's anemia.[16]

The presence of hypochromia and microcytosis in Cooley's anemia by analogy to iron and pyridoxine deficiency suggested disturbance in heme synthesis. There are, however, certain differences in these three conditions. In iron deficiency, erythrocytes contain excess protoporphyrin and the patients are generally iron deficient. In pyridoxine deficiency there is a decrease in erythrocyte porphyrin and a normal or increased supply of iron.[27] Peripheral blood studies show neither a deficiency of iron or porphyrin in Cooley's anemia.[16, 28, 29] It is possible that one metabolic defect in Cooley's anemia involving glycolysis could effect both pyrolle synthesis and cell viability. Pyrolle synthesis has been shown to be dependent on the intermediate metabolism of the Krebs cycle.[30] Abnormalities in glycolysis have been shown to be important in viability of stored erythrocytes[31] and suggested as an underlying defect in hereditary spherocytosis.[32]

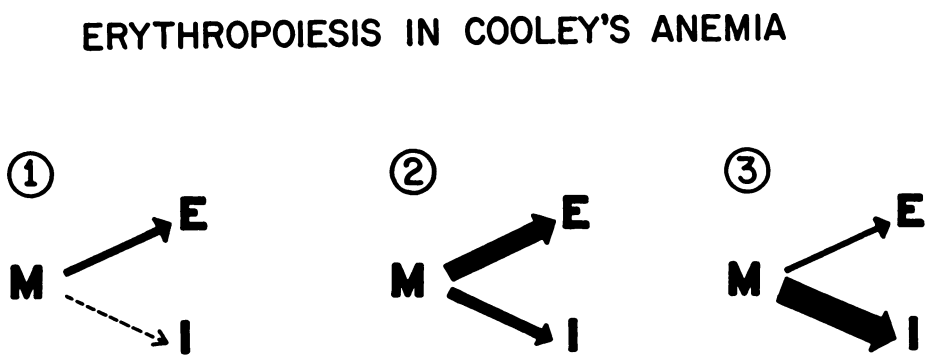

FIG. 2.—M refers to erythroid marrow, E to effective erythropoiesis and I to ineffective erythropoiesis. (1) expresses diagrammatically normal erythropoiesis . . . where nearly all production is effective, i.e., producing cells which are delivered into the circulating blood. (2) represents mild Cooley's anemia, where total erythropoiesis is greatly increased and the major portion is effective. (3) severe Cooley's anemia, where most of the marrow activity is ineffective.

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The possibility of an abnormality in carbohydrate metabolism in Cooley's anemia is also suggested by the data of Astaldi.[33]

Summary

Blood production and destruction have been measured in four patients with Cooley's anemia. Methods employed included the determination of erythroid/myeloid ratio of the marrow, reticulocyte count, plasma iron turnover and red cell utilization, Cr^{51} survival and fecal urobilinogen. Rates of production obtained by these measurements have been compared to normal.

Patients with Cooley's anemia have been shown to have an increased turnover of hemoglobin constituents comparable to the maximal response seen in other hemolytic anemias. There is, however, a marked decrease in maximal delivery of erythrocytes to the peripheral blood amounting to about 50 per cent in the mildly anemic patients and 85 per cent in severely anemic patients. The rate of destruction of circulating erythrocytes was similar in the three patients studied. The severity of anemia was therefore largely related to the production defect.

It was concluded that the defect in Cooley's anemia is not in total hemoglobin synthesis, but in the fabrication of circulating erythrocytes, which in turn have the associated manifestations of hypochromia, increased percentage of fetal hemoglobin and shortened survival time.

Summario in Interlingua

Le production e le destruction de sanguine esseva mesurate in quatro patientes con anemia de Cooley. Le methodos usate includeva le determination del proportion erythroide/myeloide del medulla, numerationes reticulocytic, metabolismo de ferro in le plasma e utilisation de illo per le cellulas rubie, superviventia cellular per etiquettage a Cr^{51}, e urobilinogeno fecal. Le valores del production que esseva obtenite per iste mesurationes esseva comparate con valores normal.

Il ha essite demonstrate que patientes con anemia de Cooley ha un augmentate metabolismo de constituentes hemoglobinic, comparabile al responsa maximal que es incontrate in altere anemias hemolytic. Il ha, nonobstante, un marcate reduction in le provision maximal de erythrocytos al sanguine peripheric. Iste reduction amonta a circa 50 pro cento in patientes qui es levemente anemic e a 85 pro cento in patientes con sever grados de anemia. Le valores pro le destruction de erythrocytos circulante esseva simile in le casos del tres patientes studiate. Ergo le severitate del anemia dependeva in grande mesura del defecto de production.

Le conclusion esseva que le defecto in anemia de Cooley non reside in le total synthese de hemoglobina sed in le fabrication de erythrocytos circulante, le quales, de lor parte, se distingue per associate manifestationes de hypochromia, un augmentate procentage de hemoglobina fetal, e un abbreviate superviventia.

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