Megaloblastic Anemia Probably Caused by Defective Utilization of Folinic Acid

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The common types of megaloblastic anemias usually respond well to vitamin B-12 or folic acid but there are some which respond incompletely or not at all to these antianemic agents.

In 1936, Israel and Wilkinson referred to these megaloblastic anemias which are refractory to liver extract as "Achrestic Anemias". Since then a number of cases have been described but only in a few instances have the underlying biochemical defects been elucidated. One example of this type of anemia is Di Guglielmo syndrome, a megaloblastic anemia the metabolic defect of which has not been defined. Wills described a few cases of megaloblastic anemias that did not respond to pure liver extract or vitamin B-12, but responded to crude liver extract. Up to the present time it is not clearly understood whether the so-called "Will's Factor" is identical with folic acid or if it is a separate entity.

Vitamin C deficiency has been observed to prevent the response to liver therapy in pernicious anemia and Vilter et al. have described megaloblastic anemia occuring in one patient with scurvy that responded to vitamin C. Holly showed that megaloblastic anemia of pregnancy refractory to vitamin B-12 responded following combined but not separate therapy with vitamin C and vitamin B-12. A combined deficiency of folic acid and ascorbic acid occurs in some cases of megaloblastic anemias of infancy. In some of these instances, it has been suggested that vitamin C deficiency may interfere with the conversion of folic acid to folinic acid.

Ungley cited the case of a patient with megaloblastic anemia without evidence of vitamin C deficiency who failed to respond to vitamin B-12, crude liver extract and folic acid but did improve on folinic acid. The metabolic defect responsible for the megaloblastic anemia was not identified. Recently, Huguley et al. reported a case of megaloblastic anemia in a 28-month old child that had massive crystalluria (orotic acid crystals) and did not respond to vitamin B-12, folic acid, pyridoxine or uracil but showed complete hemologic remission and a remarkable reduction in orotic acid excretion following the administration of a crude mixture containing uridylic and cytidylic acid while receiving steroid therapy. The authors postulated that the metabolic defect was either in ribosidation of orotic acid or in the decarboxylation of orotidylic acid to uridylic acid.

We have recently studied a patient with megaloblastic pancytopenia in which the defect in blood formation is apparently related to defective utilization of folinic acid.
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CASE REPORT

C. B., a 12 year old negro girl, was referred to Jefferson Hospital on 1-27-56. She was apparently in good health until about three weeks prior to admission when she developed multiple ulcerations of the mouth and tongue. The family history was of significance in that a sister who had died of severe anemia 10 years previously, at the age of 12, was said to have had “trench mouth” and a “hyperplastic bone marrow with a severe maturation defect”. Another sister died at the age of 5 after an illness which began with soreness of mouth and tongue. The sternal marrow findings were said to be compatible with “acute leukemia of childhood”. Bone marrow slides of the sisters were not available for study. Investigation of three living siblings revealed nothing abnormal.

At the time of admission to Jefferson Hospital, the patient appeared acutely ill, lethargic and pale. There were many small blood blisters in the mouth, pharynx and on the under surface of the tongue. Some were ulcerated and covered with thin white exudates. The cervical and axillary lymph nodes were just palpable. There was a grade 2 systolic murmur heard best at the base of the heart. There was no hepatosplenomegaly and no bony tenderness. Neurological examination revealed no evidence of nervous system disease.

Hematological data: hemoglobin, 8.01 Gm. per cent; red cell count, 2,055,000 per cu. mm.; platelets, 28,000 per cu. mm.; reticulocyte count, 0.2 per cent; and white cell count, 1700 per cu. mm., with the following differential: segmented neutrophils, 22 per cent; eosinophils, 2 per cent; lymphocytes, 68 per cent; prolymphocytes, 2 per cent; and monocytes, 6 per cent. The red cells were normochromic but exhibited moderate anisocytosis and poikilocytosis. Few neutrophils were giant with hypersegmented nuclei. The bone marrow examination showed uniform hypoplasia of all series with megaloblastic erythroid cells and giant myeloid cells. The sickling test was negative and hemoglobin electrophoresis revealed a normal pattern. The venous blood clotting time was normal in glass and in silicone coated tubes. The bleeding time was longer than 10 minutes. There was free acid in the gastric juice.

The initial clinical impression was megaloblastic anemia of childhood and folic acid therapy was instituted (on 2-3-56)—first in the dosage of 5 mg. twice daily by mouth and later increased to 20 mg. twice daily. The folic acid therapy was continued for 7 days without symptomatic or hematologic improvement. The folic acid was then discontinued and liver extract therapy started—first, 1.5 cc. of crude liver (Liver Injection Crude-Wyeth) intramuscularly for 11 days followed by 1.5 cc. of refined liver extract (Liver Injection Refined-Wyeth) daily. On the third day after cessation of folic acid and after the start of crude liver extract, the reticulocyte count rose to 4.2 per cent and reached a peak of 13 per cent on the following day. The reticulocyte peak coincided with clinical improvement and with reversion to a normal bone marrow. Eighteen days after institution of refined liver extract the reticulocyte count dropped to 0.1 per cent; the hemoglobin was 7.14 Gm. per cent; red cell count 2,310,000 per cu. mm. and the mouth ulcers began to reappear. The conclusion then was that the megaloblastic anemia was responsive to crude liver extract therapy but unresponsive to refined liver extract and most probably to folic acid.

On March 20, 1956 a Schilling test was performed and revealed urinary excretion of vitamin B-12 in excess of 20 per cent. Following this test, vitamin B-12 therapy was instituted, 30 μg. intramuscularly daily for 20 days. Two days after the Schilling test when 1000 μg. of vitamin B-12 had been administered, the reticulocyte count began to rise and reached a peak of 22 per cent on the fourth day. There was a steady rise of hemoglobin, red cell count and white cell count, but the platelet count remained at 20,000 per cu. mm. The ulcerations gradually healed and the patient was discharged on the 67th hospital day (4-4-56) on vitamin B-12, 35 μg. twice a day orally. Two weeks later and while receiving vitamin B-12 orally, she again developed small ulcerations on the margins of the tongue and soft palate. These cleared rapidly after one injection of 1000 μg. of vitamin B-12 and from then on she was given weekly injections of vitamin B-12 (1000 μg.). She did well on the regimen, and the peripheral blood studies became normal. However, it was observed that the ulcerations in the mouth recurred when the patient missed only one weekly injec-
tion. Although the patient obtained a remission with vitamin B-12 it appeared as if large dosage and continuous administration were necessary to maintain the remission.

On June 22, 1956, she began having mouth ulcers. At this time she had not received vitamin B-12 for 11 days, and the peripheral blood picture was found to be almost normal; nevertheless, the bone marrow was slightly hypocellular in all series and the red cell precursors were definitely megaloblastic. Serum vitamin B-12 level was 1200 µg. per ml. (normal: mean 300 µg. per ml. and range 100-1000 µg. per ml.) Folic acid therapy was instituted 7 mg. twice daily intramuscularly for 11 days. The reticulocyte count started to rise on the ninth day and reached a peak of 4 per cent on the fifteenth day. A marked reticulocyte response was not expected since the peripheral blood studies were almost normal at the onset of folic acid therapy. The mouth ulcers healed and the bone marrow was found to be normal 14 days after institution of therapy. Patient maintained remission until October 24, 1956 when ulcers reappeared in the mouth. The striking feature of the folic acid response compared to the previous responses was the maintenance of remission for 78 days without treatment. The impression at this time was that the formation of folinic acid from folic acid was defective and that this defect rather than folic acid and vitamin B-12 deficiency was responsible for the megaloblastic anemia.

On October 25, 1956 combined oral therapy of folic acid (10 mg. daily) and vitamin C (500 mg. daily) was started. Blood studies showed a hemoglobin of 7.92 Gm. per cent; red cell count, 2,750,000 per cu. mm.; reticulocyte count, 0.2 per cent; platelet count, 124,000 per cu. mm.; white cell count, 2400 per cu. mm.; and a hypercellular megaloblastic bone marrow. Vitamin C level in plasma prior to therapy was found to be normal (2–3 mg. per cent). She responded well clinically, her mouth ulcers disappeared, the reticulocytes began to rise on the tenth day of treatment reaching a peak of 22 per cent on the nineteenth day and in the third week, the bone marrow was normoblastic. It was felt that remission could be induced and maintained by folic acid and vitamin C. In order to evaluate the drugs separately, folic acid therapy was stopped on December 19, 1956, and for 36 days she received 300 mg. of vitamin C daily by mouth. Nineteen days after the cessation of folic acid, she developed ulcerations in the mouth and the bone marrow was megaloblastic. Patient received no treatment till early in March, 1957 when a full blown clinical (mouth symptoms, fever and mental apathy) and hematologic (megaloblastic bone marrow; red cell count, 1,500,000 per cu. mm.; hemoglobin, 4.9 Gm. per cent; reticulocyte count, 0 per cent; white cell count, 1,850 per cu. mm.; and platelet count 32,000 per cu.mm.) relapse was induced for the determination of blood and urine levels of folinic acid before and after therapy with folic acid and a combined therapy with vitamin C. On March 11, 1957 folic acid alone 15 mg. daily was administered for 14 days (fig. 1) without a response. This confirmed our previous impression that folic acid therapy alone was not effective. Her condition on 3-23-57 was such that it necessitated transfusion of 250 cc. of packed red cells. On March 25, 1957 vitamin C (200 mg. daily) therapy was added (both drugs were administered intramuscularly). On April 3, (nine days after the onset of the combined therapy) the reticulocyte count rose to 5.2 per cent and two days later reached a peak of 24.2 per cent. The fever abated and patient became progressively better. Eventually a complete hematologic remission was obtained. Figure 1 depicts the urinary daily excretion of folinic acid. Unfortunately all the blood specimens saved for the folic acid determination were lost. During relapse and prior to the medication the urinary excretion of folinic acid was 3–10 µg. per day (normal value is 1–2 µg., Dr. Broquist¹). It rose to 40–50 µg. per day after the administration of folic acid. With combined therapy of folic acid and vitamin C the urinary level of folinic acid rose to 142.6–396 µg. per day. The same studies were repeated before and after homocysteine therapy. In July, 1957 folic acid and vitamin C therapy was discontinued and about the end of August patient entered a partial relapse manifesting mouth ulcers and megaloblastoid bone marrow. The peripheral blood picture was almost normal. On the eighth of September, 1957 homocysteine (50 mg. per day) was administered intramuscularly for six days without an increase in the urinary excretion of folinic acid. Four days later folic acid 15 mg. per day intramuscularly was given with homocysteine. Two days after this
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Fig. 1.—Urinary folic acid values and hematological data before and after treatment with: (1) Folic Acid, (2) Folic Acid and Vitamin C, (3) Homocysteine, (4) Folic Acid and Homocysteine.

Combined therapy had been started there was significant rise in the 24 urine output of folic acid. Soon thereafter, the mouth ulcers disappeared and the bone marrow became normal.

In the ensuing two years serum folic acid level was determined on two occasions one week and two weeks respectively after cessation of treatment with folic acid and homocysteine and was 0.3 μg/ml on both occasions. (Normal sera values: 1.9, 0.6, 0.8, 0.6, 0.7, and 0.4 μg/ml, Dr. Hendlin*). After re-institution of previous therapy the level of serum folic acid increased to 5.2 μg/ml. In order to test for naturally occurring antifolic acid agent in the patient, experiments were carried out to attempt recovery of added folic acid to the urines of the patient and two other controls (one was normal and the other, a patient with acute leukemia receiving Methotrexate) and the serum of the patient. The results indicated complete recovery of added folic acid only with the patient's serum, urine and the normal urine.

For the last 24 months, patient has been on a combined therapy of folic acid 15 mg. per day and homocysteine 25 mg. per day (both orally) and has maintained a complete clinical and hematologic remission. On two occasions when folic acid unintentionally was given alone for a period of three weeks she developed transient mouth ulcers.

Discussion

The main action of folic acid as determined in tissue and bacterial systems seems to involve the transfer of 1-carbon units among conjugate pair of substances: glycine and serine, carboxamidase and purine, uracil ribotside and thymine ribotide, homocysteine and methionine and amino-ethanol and chol...

*Note: The folic acid determinations were obtained by the Leucostoc citrovorum assay method. Leucovorin Lederle was employed as standard and the values reported were corrected for the inactive isomer. The procedure is published elsewhere.
The same action is also utilized in the biosynthesis and breakdown of histidine\(^{12}\) (fig. 2). Folic acid after conversion to tetrahydrofolic acid, the coenzyme, acts as a carrier of the 1-carbon unit which is in the form of "active formyl". The combined product, producing N\(^5\) formyl tetrahydrofolic acid, is better known as folinic acid. This is isomerized to N\(^10\) formyl tetrahydrofolic acid which is capable of donating its active formyl group to various acceptors.\(^{12}\) The in vivo conversion of folic acid to folinic acid\(^{13,14,15}\) and the demonstration that folinic acid can reverse aminopterin toxicity more effectively than folic acid\(^{16,17}\) have given support to this concept. The conversion of folic acid to folinic acid occurs in the following steps: First, folic acid is reduced to dihydrofolic acid by a reducing agent; vitamin C\(^{18,19,20}\) or homocysteine.\(^{21}\) Second, dihydrofolic acid is reduced further by dihydrofolic reductase to tetrahydrofolic acid.\(^{22,23}\) This enzyme is inhibited by antifolic compounds.\(^{23}\) The reduced form of triphosphopyridine nucleotide (TPNH), in both steps, provides the reducing power. The third step is the addition of a formate group to tetrahydrofolic acid from a formate source (e.g., serine). Earlier work\(^{24,25}\) has implicated vitamin B-12 in the conversion and even utilization of folinic acid.

Recent reviews\(^{26,27}\) of the metabolic role of vitamin B-12 suggest that it is necessary for labile methyl group synthesis from 1-carbon precursors. For example, vitamin B-12 is necessary for reduction of the formate in uracil to thymine methyl. This action is complementary to the previously mentioned action of folic acid. Currently, it seems that the transformation of uracil nucleotide (after it is formed from orotic acid\(^{28,29}\) to thymine nucleotide) is the main site of action of vitamin B-12 and folic acid in the biosynthesis of pyrimidine nucleotides.

The patient described here is a 12 year old negro girl, who presented her-
self with megaloblastic anemia, leukopenia, thrombocytopenia, mouth ulcers, excessive salivation, loss of appetite and lethargy. The presence of free acid in the gastric juice, normal serum vitamin B-12 level and normal Schilling test ruled out a vitamin B-12 deficiency or juvenile pernicious anemia. Normal bowel functions as indicated by the history, radioactive fat absorption studies and later by lack of response to folic acid (fig. 1) appear to preclude a malabsorption state. Megaloblastic anemia of infancy was not likely; the patient was 12 years old and had normal vitamin C blood levels. In addition patient failed to respond and maintain a remission on folic acid or vitamin C.

In an attempt to study the underlying cause, patient was treated with several antianemic agents. Initially, although not conclusive, there seemed to be no response to folic acid. Crude liver extract induced a remission but purified liver extract failed to maintain the remission which lasted approximately three weeks. Vitamin B-12 was capable of inducing and maintaining a remission; however, it had to be given in exceedingly large weekly doses (1000 µg a week). Mouth ulcers recurred 11 days after cessation of vitamin B-12 therapy and the bone marrow became megaloblastic in 2 weeks. We felt that vitamin B-12, although beneficial to the patient, did not elicit the same kind of response one sees in pernicious anemia. Vitamin B-12 may possibly have acted by enhancing the formation and utilization of folinic acid. Subsequent administration of folinic acid in large doses over an 11-day period induced a remission that lasted 78 days, the longest period in which the patient could maintain a remission without therapy. This suggested strongly that the patient was unable to convert folic acid to folinic acid and that her tissues were capable of storing folinic acid. However, the observations were not conclusive since folinic acid was administered in pharmacologic amounts.

To study further the conversion of folic acid to folinic acid, the patient received a combined therapy of folic acid and vitamin C which induced a complete clinical and hematologic response. The clinical remission lasted 2–3 weeks and the bone marrow became megaloblastic in one month after discontinuation of therapy. Similar observations were made when the combined therapy was changed to folic acid and homocysteine. Homocysteine and vitamin C were administered both orally and intramuscularly with the same response obtained. Since September, 1957, patient has been receiving folic acid and homocysteine and has been maintained in a complete remission. The reason for continued use of homocysteine instead of vitamin C in the combined therapy is the observation made by the mother that the behavior of her daughter appears to be better on homocysteine.

It was suggested that the defect in the conversion of folic acid to folinic acid perhaps was due to the presence of a naturally occurring antifolic compound since the patient presented a clinical picture reminding of the toxic manifestations of antifolic agents (mouth ulcers, excessive salivation and loss of appetite) and since these agents are considered to inhibit the formation and utilization of folinic acid. Recovery experiments with the patient's urine and serum, using the Leuconostoc Citrovorum-assay method, showed complete recovery of added folinic acid. This most probably precluded the presence of a naturally occurring antimetabolite.

The hypothesis that the underlying defect was related to formation of folinic
acid was examined further by means of folinic acid determination in the blood and the urine of the patient during relapse and after administration of folic acid, combined folic acid and vitamin C, homocysteine and combined folic acid and homocysteine (fig. 1). The results of the urinary folinic acid determinations were unexpected. The urinary excretion of folinic acid during relapses was slightly high between 3-10 μg. per day (normal 1-2 μg. per day) and rose to almost expected values (40-50 μg. per day) after administration of folic acid18 and yet there was no clinical or hematologic response. It was found after administration of folic acid and vitamin C (or homocysteine) that the patient obtained and maintained a complete remission. Then the urinary excretion of folinic acid was exceedingly high (142.6-396 μg. per day and 130-139 μg. per day respectively). These determinations did not support the hypothesis that formation of folinic acid was defective but they suggested that utilization rather than formation of folic acid was at fault.

Folinic acid level in serum was determined on two occasions after cessation of treatment with folic acid and homocysteine and the results revealed a low normal level (0.3 μg. per ml.) This level increased to 5.2 μg. /ml. after the reinstitution of previous treatment. The apparent discrepancy between the borderline low to low normal serum level and the slightly increased amount of urinary folic acid during relapse could be explained on the basis of an obligatory renal loss and perhaps an induced change in renal excretion in the face of poor utilization of folic acid. This situation is similar to that of folic acid in relation to fasting.31 megaloblastic anemia of cirrhosis and tropical sprue. Herbert and co-workers32 have found low serum level of folic acid in patients with cirrhosis and megaloblastic anemia. In the same disorder, Jandl and Lear33 observed almost normal urinary excretion of folic acid in some of their patients. Butterworth and co-workers34 studies in patients with tropical sprue revealed continued excretion of folic acid, folic acid and related compounds even during relapse.

The possibility of a genetic defect is suggested by the fact that one sister died of severe anemia associated with “trench mouth” and a hyperplastic bone marrow with a maturation defect and another sister died of “acute leukemia of childhood” which began with soreness of mouth and tongue. Unfortunately, slides of the bone marrow of those two sisters were not available.

**Summary**

A 12 year old negro girl was studied who presented a picture of megaloblastic anemia refractory to physiologic doses of vitamin B-12 and to folic acid. However, remission could be induced and maintained by pharmacologic doses of vitamin B-12 (1000 μg. per week), by folic acid or by a combination of vitamin C (or homocysteine) and folic acid. Folinic acid determinations and the clinical response to various metabolites indicated the existence of a metabolic defect in the utilization of folic acid. The hereditary nature of this defect was suggested by the fact that two siblings might have had a similar condition.

**Summario in Interlingua**

Essa studiata un puera negre de 12 annos de etate qui presentava un tableau de anemia megaloblastic refractori a doses physiologic de vitamina
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B-12 e acido folic. Tamen, remission poteva esser inducite e mantenite per doses pharmacologic de vitamina B-12 (1000 µg per septimana), per acido folinic, o per un combination de vitamina C (o homocysteina) con acido folic. Determinationes de acido folinic e le responsa clinic a varie metabolitos indicava le existentia de un defecto metabolic in le utilisation de acido folic. Le natura hereditari de iste defecto esseva suggerite per le facto que duo fratemos habeva possibilemente unsimile condition.

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REFERENCES


Intra-erythrocytic crystals similar to those described in disorders associated with Hb.C, were found in Hb.E-thalassemia disease, Hb.E trait, thalassemia trait and homozygous thalassemia—J. B. C.


The urinary excretions of different nitrogenous constituents, such as non-protein N, urea N, uric acid, amino acid N, creatinine and creatine, were estimated during the process of the combined vitamin deficiencies. All the nitrogenous constituents except creatine were excreted in increased amounts in vitamin-deficient animals. Glucose, total protein and nonprotein N content of blood and total protein contents of liver were determined in normal and deficiency animals. No change in protein content of blood and liver could be obtained in the deficient animals. The nonprotein N of blood was high and the fasting blood sugar level was low in the deficient animals.—J. B. C.
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