Studies of Folate Deficiency in Patients with Neoplastic Diseases


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SUMMARY

Seven patients with advanced neoplastic diseases were fed a semisynthetic folate-deficient diet for periods ranging from 25 to 140 days. A rapid drop in serum folate was observed in all 7 patients within the first 10 days of feeding with the formula diet. A slower drop in whole-blood folate became apparent after 30 days. There was a substantial drop in liver folate in 4 out of 5 patients, in tumor folate in 4 out of 4 patients, and in isolated leukocyte folate in the 1 patient tested. Macrocytosis developed in 1 patient after 132 days of feeding with the deficient diet. Slight megaloblastoid changes in the bone marrow were observed from Day 91. Both the macrocytosis and megaloblastoid changes reverted to normal upon resumption of a regular diet and folate supplementation. The rate of folate depletion in the tumor tissue was slow and roughly paralleled the rate of depletion in liver and blood. It was concluded that folate depletion by dietary means is an impractical approach to the treatment of human cancer.

INTRODUCTION

Tetrahydrofolate cofactors are required for the de novo synthesis of inosinic acid and thymidylylate. This requirement makes folic acid an essential nutritional factor for growth and division of cells including neoplastic cells. Folic acid antagonists are effective in the treatment of certain human neoplastic diseases, but clinical resistance to these agents eventually develops in most cases. Definite growth inhibition of Walker 256 carcinosarcoma in rats has been reported when animals were fed a folate-deficient diet supplemented with 4% Sulfasuxidine (9). This transplantable tumor is known to be refractory to administration of the folic antagonist methotrexate (9). These apparently conflicting observations have been interpreted as being due to common pathways of cellular uptake for vitamin and drug. The same inefficient pathway which protects against drug effect makes the cell unable to compete successfully for declining levels of circulating vitamin (9). This observation suggested a possible way to circumvent natural and possibly acquired tumor resistance to antifols in man, that is, the induction of folate deficiency by dietary means.

The experience with attempts to induce folate depletion by dietary means in 7 patients with neoplastic disease is the subject of this paper.

MATERIALS AND METHODS

Each of the 7 patients studied had a histologically proven active neoplastic disease with measurable parameters for proper evaluation of the effects of treatment. All but one of the patients had an estimated survival of not less than 3 months, and they all agreed to a tentative 3-month hospitalization period. The patients were chosen from among the hospital patients when their disease became refractory to other forms of treatment or when no known curative treatment was available.

During a period of about 1 week the patients were allowed their regular diet and their daily caloric intake was estimated. Base line studies were performed at this time. They included the following hematological tests: hemoglobin concentration, hematocrit, red cell, and reticulocyte counts; white cell and differential counts, and platelet counts; mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and bone marrow aspiration. Arneth counts were performed on 4 patients. The following clinical laboratory tests also were performed: blood urea nitrogen, serum uric acid, total protein and albumin, alkaline phosphatase, total and free cholesterol, bilirubin, glutamic-oxalacetic transaminase, cephalin flocculation, prothrombin time, electrolytes, and serum iron. On all patients serum folate activity was performed with a slight modification of the method reported by Romine (8). The following clinical laboratory tests also were performed:

blood urea nitrogen, serum uric acid, total protein and albumin, alkaline phosphatase, total and free cholesterol, bilirubin, glutamic-oxalacetic transaminase, cephalin flocculation, prothrombin time, electrolytes, and serum iron.

On all patients serum folate activity was performed with a slight modification of the method reported by Waters and Mollin (10), and folic acid measurement in whole blood (3) was performed on 5 patients. Measurement of folate activity on liver samples obtained by needle biopsy from 5 patients and on tumor samples on 4 patients were also performed with a slight modification of the method reported by Romine (8). After the caloric intake of each patient was estimated and the base line studies were performed the planned treatment was started. Patients were kept in a clinical research center under “metabolism discipline.” The treatment consisted of feeding the patients a semisynthetic liquid formula diet deficient in all vitamins except vitamin E. The diet was supplied by Mead Johnson and Company (Evansville, Indiana) in powder form in sealed cans each containing 147 g of diet formula (700 calories) (Table 1). The contents of

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each can were blended in about 400 ml water on the day of use, then divided into aliquots, and refrigerated. The patients were usually given 6 feedings a day between 8 a.m. and 10 p.m. All other foodstuffs except tea or coffee with sugar or saccharine were excluded. Vitamins other than folate were supplied daily in adequate amounts by 2 chewable, specially formulated folate-free multivitamin tablets prepared for this study by Mead Johnson and Company (Table 1).

Seven patients were studied, 3 women and 4 men ranging in age from 34 to 77 years (Table 2). The patients were fed the folate-deficient diet for 25 to 140 days. The biochemical and hematological studies, except for marrow aspirations, were repeated every 1 to 2 weeks during the study period. Bone marrow aspirations and liver and tumor biopsies were repeated at 1- to 2-month intervals on patients remaining on study. Folate clearance tests (1) were done at the conclusion of the study in 1 patient.

### Measurement of Folate Activity

**Sample Preparation.** Blood from fasting subjects was transferred to a sterile test tube and allowed to clot at room temperature for approximately 3 hr. The samples were then centrifuged at 3000 rpm for 5 min. Ascorbic acid (0.5%, w/v) was added to the serum for preservation of folate activity, and the samples were frozen until assay. Whole blood was prepared and frozen with the use of the procedure of Grossowicz et al. (3).

Liver biopsy samples were homogenized in a Potter-Elvehjem homogenizer in a concentration of 2 to 10 mg, wet weight, per ml 0.1 M potassium phosphate buffer, pH 7.0, containing 1% ascorbic acid. Samples were sonically treated for 2 min and autoclaved for 2.5 min at 15 psi. The samples were centrifuged at 3000 rpm for 5 min and the supernatant was frozen. The procedures were the same for tumor tissue except for dilution to 20 to 100 mg/ml. Leukocytes were separated (2) and diluted to 1 x 10⁸ cells/ml buffer prior to homogenization.

**Assay Procedure.** Lactobacillus casei (ATCC 7469) was the organism used to measure total folate activity. The bacilli were maintained in 5 ml basal medium (10) with 5 μg folic acid added. The assay was carried out in acid-washed Klett tubes (13 x 125 mm) with 2 ml double-strength basal medium; 0.5 M sodium phosphate buffer, pH 6.1; and ascorbic acid so that each tube contained 5 mg ascorbic acid after addition of samples or standard preparation. Water was added to make the total volume in each tube 4 ml.

A stock solution containing 10 μg folic acid/ml was prepared and frozen. A standard growth curve was prepared by diluting the stock solution to concentrations of 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 μg/tube. Each concentration was set up in triplicate. After all components,
except the samples, were added to the tubes they were covered with aluminum foil and autoclaved for 10 min at 110° and 10 psi. Three concentrations of each sample were assayed in triplicate and diluted so that 0.05, 0.1, and 0.2 ml contained between 0.2 and 2.0 mg folate activity. All samples were added aseptically. Usually, serum, whole-blood hemolysates, and other tissue homogenates could be added without further dilution if prepared as stated above.

Inoculum. A 24-hr L. casei culture was washed 3 times with sterile 0.9% NaCl solution and diluted to read 30 Klett units against a blank of the same solution. To 0.5 ml of this dilution, 9.5 ml 0.9% NaCl solution were added and 1 drop of this solution was added to each tube except to 1 "zero" concentration tube from the standard curve and 1 tube from each concentration of each sample which served as uninoculated blanks. All tubes were shaken and incubated at 37° for 18 to 24 hr or until the highest concentration of the standard curve measured 180 to 200 Klett units. Tubes were then thoroughly shaken and read in a Klett-Summerson colorimeter with a No. 66 red filter. The standard curve was plotted on semilogarithmic paper. Multiple dilutions of unknown sera were shown to have identical slope with the standard curve. Those sample dilutions falling within the straight line (logarithmic) portion of the standard curve were averaged to determine folate concentration of samples. With this method serum folate concentration measured on 23 normal adults ranged from 3.6 to 9.9 μg/ml with a median of 5.5 μg/ml.

RESULTS

The most complete and detailed study was carried out on a 63-year-old city employee (Patient 1, Table 2) who had a history of a left postauricular sore for 15 years. When the patient was first seen, 1.5 years prior to folate deficiency treatment, the left auricle was destroyed; a necrotic infiltrating ulcer, 3.5 x 4 cm in diameter, was present; and the patient had left facial paralysis. After radiotherapy with 3775 rads followed by treatment with Meturedepa (AB-132) and intratumor equine respiratory virus injection, he sustained healing and remission for 10 months. Local recurrence of the tumor and ulceration prompted admission to our service.

The folic acid deficiency study was presented to the patient and accepted. In addition to the standard base line studies, a Schilling test was performed which showed 14% excretion in 24 hr. The patient was fed the vitamin-deficient diet for a total of 140 days. During this period he received 2 folic acid-deficient multivitamin tablets daily. His daily caloric intake was, with minor variations, 2100 calories. His appetite was good and he vomited only once on Day 137. His weight was 68.1 kg on Day 140 compared to his base line weight of 69.6 kg, a loss of 1.5 kg. No appreciable change in the clinical findings took place during the 140-day study period. He remained cheerful throughout the dietary treatment and was active within the confines of the metabolic ward. During the study period he received no systemic antibiotic treatment, but neomycin ointment was applied to the left auricular ulcer sporadically during the first 61 days of treatment. The ointment was discontinued altogether in the last 80 days of the study to ensure that the very low serum folate levels were not a consequence of absorption of antibiotics interfering with the microbiological assay.

Assay for folate activity of serum, whole blood, packed leukocytes, and tumor and liver biopsy specimens were performed prior to the initiation of the study and at variable periods during the study. At the onset of the study the serum folate activity was low (3.3 μg/ml) and slowly declined to a nadir of 0.35 μg/ml by Day 127 (Chart 1). Similarly, there was progressive decline in folate activity of whole blood. Liver folate activity declined by half after 47 days of the deficient diet. Further marked decline took place by Day 91. Tumor folate levels showed a similar decline. The final liver and tumor biopsies on Day 137 showed a slight but probably insignificant increase of folate activity compared to Day 91 (Chart 1). There was no evidence of breach in the dietary discipline. Folate activity in packed isolated leukocytes declined from 37 μg/10⁹ cells on Day 12 to 2.75 μg/10⁹ cells on Day 137 (Chart 1).

A folate clearance study was done on the last day of study and was repeated on Days 1, 3, 6, and 12 in the poststudy period. Folic acid, 1 mg, was injected intravenously and the serum was assayed at 3, 15, and 30 min for folate activity.

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**Chart 1.** Measurement of folate activity in liver in μg/g (●), tumor in μg/g (●), blood in μg/ml (○), isolated leukocytes in μg/10⁹ cells (●), and serum in μg/ml (●) in Patient 1 during the period of feeding with folate-deficient diet.
In the first clearance study the serum folate activity dropped to 11 μg/ml at 30 min which indicates increased clearance (88% at 30 min) consistent with folate deficiency (Chart 2). Subsequent folate clearance studies were 73, 77, 77, and 49% on Days 1, 3, 6, and 12, respectively.

There was a progressive drop in hemoglobin concentration from a base line of 13.1 to 9 g/100 ml on Day 140. The packed red cell volume of 40% and red cell counts of 4.3 millions/μl dropped to 29% and 3.09 millions/μl by Day 140. The reticulocytes fluctuated from 0.2 to 1.4% throughout the depletion period. The mean corpuscular volume ranged around 88 cu μ for 123 days. Thereafter, the MCV increased to 101 cu μ on Day 132 to a maximum of 102 cu μ on Day 140 (Chart 3). The mean corpuscular hemoglobin followed a pattern similar to that of MCV, ranging around 28 μg for 123 days, then rising to 34.7 μg by Day 140. The mean corpuscular hemoglobin concentration fluctuated within a narrow range of 31 to 33%. There were wide variations in the serum iron levels throughout the study period from 38 to 280 μg/100 ml, but the levels were mostly within the normal range of 80 to 130 μg/100 ml.

No definite change in the peripheral leukocyte count was noted. Some increase in the number of segments in the neutrophils occurred from a mean of 3.2 lobes/cell in the first 112 days of study to a mean of 4.1 lobes/cell in the last 28 days of study. No definite change in platelet counts was noted. Bone marrow aspirate on Day 91 showed megaloblastoid changes in some cells. The marrow on Day 137 showed further increase in the abnormality with some dissociation of the cytoplasmic and nuclear maturation but no classic megaloblastic change.

After a regular diet and folic acid administration was instituted, the reticulocyte count reached a maximum of 2.6% (Chart 3). Beginning on the 7th poststudy day, 300 mg ferrous gluconate was given daily without further increase in the reticulocyte count. The hemoglobin concentration rose slowly to 11.6 g/100 ml by the 24th day of the poststudy period. This was accompanied by a gradual decrease of the MCV to 88.2 cu μ and of the reticulocyte count to 0.2%. Bone marrow on Day 28 poststudy showed a disappearance of the megaloblastoid changes. Serum folate on the 20th poststudy day and the tumor folate on the 40th poststudy day had returned to the predeficiency levels.

The other 6 patients were fed the deficient diet for briefer periods from 25 to 95 days (Table 2). None had any tumor regression during this period; definite tumor progression was evident in 2 of the patients. Two patients died during the study period. Their deaths were caused by advanced cancer in one and secondary hemorrhage in the other. The study was discontinued on Day 28 in a patient with metastatic breast cancer when she became depressed, lost her appetite, and asked termination of the study. The study was discontinued on Day 63 in another patient with reticulum cell sarcoma when she became severely depressed at the evidence of progressive disease despite continuous treatment.

None of the patients developed any skin or mucous membrane changes or gastrointestinal or neurological signs of symptoms that could be attributed to folate deficiency.

All the patients had a drop in hemoglobin concentration with a corresponding decrease in red cell counts and hematocrits. This could not be ascribed to folic acid deficiency because there was a rough correlation of anemia with the sickness of the patient, marrow invasion by tumor, present and past radiation therapy, former chemotherapy, and the complication of considerable blood letting for studies. Significant changes or consistent trends in reticulocyte, granulocyte, lymphocyte, or platelet counts were not observed. Arneth counts on blood smears were done on 4 patients; only 1 patient (Patient 1; discussed above) showed some increase in the mean number of segments of neutrophils.

There was a rapid drop in the serum folate activity within the first 20 days of the depletion period from a median of 6.2 μg/ml at the base line period to a median of 2.1 μg/ml on Day 20. The rate of drop was much slower thereafter, although the downward trend continued (Chart 4). The folate activity of whole blood was measured in 5 patients. No apparent decrease in blood folate activity took place in the first month of the study. A drop was noticed by Day 40 and the downward trend continued in the 2 patients studied beyond 1 month (Chart 5) even after correction for the drop in hematocrit. Liver folate activity decreased in 4

\[2\] The abbreviation used is: MCV, mean corpuscular volume.
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Chart 3. Reticulocyte percentage (○) and mean corpuscular volume measurements (●) in Patient 1 during the period of feeding with folate-deficient diet and after administration of folic acid (arrow).

Chart 4. Serum folate (mean of measurements in preceding 10 days in individual patients) during the period of feeding with folate-deficient diet.

Out of 5 patients (Chart 6) and tumor folate in 4 out of 4 patients (Chart 7).

DISCUSSION

Folate depletion by dietary means is an impractical approach to the treatment of human cancer. The process of depletion was slow and, at least in the spectrum of tumors tested, the rate of folate depletion in the tumor roughly paralleled the rate of depletion in the liver and whole blood.

This study confirms the observation of Herbert (4) of the feasibility of inducing folate depletion in man by dietary means. A drop of liver folate activity took place in 4 out of 5 patients. A parallel drop was noted in tumor folate activity in 4 out of 4 patients. There was no appreciable drop in whole-blood folate activity in the first month of the depletion period (Chart 5). This is explained by the observation that mature red cells are relatively impermeable to folate (5) and as a consequence they maintain folate throughout their life span.

In Patient 1 a marked drop in the liver folate was observed by Day 91. This time coincided with the appearance of slight megaloblastoid changes in the erythroid series in the bone marrow. These changes preceded the rise in MCV by 41 days. A more definite megaloblastoid marrow morphology was seen at Day 137. The blood and marrow changes occurred at about the same time as in the case reported by Herbert (about Day 135) (4). The MCV was
higher in the present case than in Herbert's (102 cu μ compared to 93 cu μ) although the megaloblastic changes were less prominent. The rise in liver folate in Patient 3 from a base line of 4000 μg/g to 4700 μg/g by Day 83 cannot be explained. The possibility of inadvertent administration of folate is extremely remote. The serum and whole-blood folate fell by more than 60%. The possibility of redistribution of folate in the body must be considered, for the hepatic rise was coincidental with a marked pulmonary and osseous tumor regression induced by X-ray irradiation.

A rapid drop in the serum folate activity became apparent within the first 10 days in all patients. This is similar to the experience of Herbert (4). The rate of drop declined after the first 20 days, although a more or less progressive drop continued throughout the depletion period. Of the 7 patients 4 had a serum folate activity of less than 2 μg/ml by Day 30 while the whole-blood folate activity remained unchanged during that period of time and the liver and tumor folate activity showed only a modest drop. The data show, therefore, that serum folate is a relatively poor index of total-body folate stores. During the folate starvation the serum folate dropped to very low levels in the absence of signs or symptoms of folate deficiency and in the presence of abundant liver and tumor folate activity. This phenomenon could explain some of the reported low serum folate levels in certain disease states (6, 7). The low levels may be attributable to temporary malnutrition but they do not necessarily reflect tissue deficiencies.

The decrease in hemoglobin concentration, increase in MCV, and the appearance of megaloblastic changes in the marrow in Patient 1 after 91 days of feeding with the deficient diet and the gradual reversion of these changes after folate administration present good evidence that these changes were caused by folate deficiency and confirms the experience of Herbert (4). A Schilling test was normal at the beginning of the study, and the patient was given 6 μg
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Chart 7. Tumor folate levels during the period of feeding with folate-deficient diet in Patients 1 (○), 5 (○), and 6 (●), and 7 (X). Effect of feeding with regular diet and folate supplementation on tumor folate level in Patient 1 is also shown.

vitamin B₁₂ orally daily. Furthermore, the daily iron intake was 22.5 mg and although the serum iron showed variations it was usually within normal limits.

The spectrum of human tumors tested seems to differ qualitatively from Walker 256 carcinosarcoma. The Walker carcinosarcoma was incapable of competing successfully with the liver for folate upon feeding with the folate-deficient diet (9). All the tumors tested compete equally well for folate in the same patient. Thus, although some human tumors theoretically may be similar to the Walker 256 carcinosarcoma, none was found in this small series. Although interest might center on patients with acute leukemia ordinarily refractory to folic acid antimetabolites, design and conduct of a study to fit this proposition has been difficult in view of the time needed to attain deficiency.

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