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Abstract

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FOLIC ACID METABOLISM IN RAUSCHER MURINE LEUKEMIA AND EFFECT OF METHOTREXATE ON THE BLOOD PICTURE

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Abstract: Folic acid contents of plasma, whole blood, liver and spleen in Rauscher leukemic mice were estimated. Plasma and liver folate in the leukemic mice was lower than that of normal mice, suggesting that folic acid was deficient in Rauscher leukemia. Folic acid contents in whole blood and spleen were even higher in the leukemic mice than those in normal mice. Clearance study by injecting folic acid intravenously into leukemic mice showed faster disappearance of folic acid from the circulating blood, suggesting that folic acid demand of Rauscher leukemia is increased. Methotrexate administered shortly after inoculation of the virus did not prevent Rauscher leukemia. But anemia, reticulocytosis and erythroblastosis, which are commonly seen 3-4 weeks later in leukemic controls, were not marked as compared with controls. It can be concluded that the requirement of folic acid is greater in Rauscher leukemia than in controls, and methotrexate is effective for preventing hematological changes commonly seen in this type of leukemias.

Folic acid is a cofactor of thymidilate synthetase during the synthesis of DNA (1), and it plays an important role in cell division through its action (2) in the DNA synthesis. Its requirement increases in malignant neoplasms and leukemias, because cell division is accelerated in such diseases (3). The fact that a folic acid antagonist, amethopterin (methotrexate, MTX) is effective for some leukemias and choriocarcinoma (4) suggests that folic acid metabolism is accelerated in these diseases.

Prolongation of the survival time in the animals with leukemias by MTX has been reported (5) (6). But folic acid metabolism in experimental leukemias of mice is little known except a report of silverman *et al.* (7); and SOTOBAYASHI (8) who studied folic acid metabolism in Walker sarcoma.

In the present paper, folic acid metabolism and effects of MTX are examined in Rauscher leukemic mice to study the relationship between folate

metabolism and malignant neoplasms.

MATERIALS AND METHODS

1) *Mice and preparation of standard virus*:

Mature BALB/c mice, weighing 19-25 g and maintained in this laboratory, were inoculated with Rauscher virus. The virus was obtained from Dr. FRANK J. RAUSCHER, National Cancer Institute, Bethesda, Maryland and has been maintained in this laboratory.

Preparation of virus suspension was as follows; spleens with Rauscher leukemia were homogenized in a chilled mortar and suspended to 10% by weight in 0.5 M sodium citrate. This suspension was centrifuged at 1800×g for 20 min and the supernatant was recentrifuged at 5000×g for 20 min.

2) *Virus inoculum*:

A frozen aliquot of concentrated virus was diluted with an equal amount of sterile 0.9% saline, and 0.2 ml of the diluted virus perparation was inoculated into mice intraperitoneally.

3) *Folate assay of plasma, whole blood, liver and spleen*:

Blood was taken by heart puncture three to four weeks after virus inoculation. The microbiological assay was made using *Lactobacillus casei* as test organism. The method for assaying plasma and whole blood were based on HARPER *et al.* (9) and HOFFBRAND *et al.* (10) respectivelly. Extraction of folic acid from the liver and spleen was as follows. Five hundred mg of the liver or spleen tissue was homogenized by adding 10 ml of 0.1 M phosphate buffer (pH 6.0 and 150 mg/dl ascorbic acid added). The spleen of the normal mice was homogenized as a whole because its weight was below 200 mg. The homogenates were autoclaved for 15 min at 121°C and centrifuged at 2000×g. Free folate was assayed after diluting the supernatant adequately by the method of WATERS and MOLLIN (11). The supernatant for assaying total folate was incubated at 37°C for 24 hr after the addition of one ml conjugase solution (3mg/ml dessicated chicken pancreas "Difco") per 10 ml of the supernatant. One drop of toluene was poured onto it to avoid the contamination by microorganism. Total folate from the conjugase-treated supernatant was assayed by the same method as that described in the case with free folate. Folic acid content of the conjugase after incubation for 24 hr was assayed. Its mean value was about 400 ng/g and this amount was negligible because folic acid contents in the liver and spleen were much greater.

4) *Folic acid tolerance test*:

Pteroylglutamic acid (40 μg/kg) was injected into the mice from the tail vein. Folate levels in plasma obtained at 15, 30 and 60 min after injecting folic acid were estimated microbiologically with four leukemic mice and five controls.

5) *Effect of MTX during the development of Rauscher leukemia*:

The virus was innoculated into 18 mice. MTX (2mg/kg) was injected into 9 mice intraperitoneally twice a week after five days of the innoculation of the virus for four weeks. To the remaining 9 mice, 0.5 ml physiologic saline was

injected as controls. Peripheral blood obtained by incising the tail of mice was examined before and two, three and four weeks after first injection of MTX. Bleeding from the wound was not marked. The examination included red blood cells, nucleated cells, reticulocytes, hematocrit and differentials of white blood cells.

RESULTS

1. *Folic acid contents in plasma, whole blood, liver and spleen* (Table 1):

TABLE 1 FOLIC ACID CONTENTS OF BLOOD AND TISSUES OF RAUSCHER LEUKEMIC MICE

	Plasma*	Whole blood*	Liver** Free	Liver** Total	Spleen** Free	Spleen** Total
Rauscher mice	38.7±13.3 (19)	651.1±346.0 (19)	5.8±2.3 (23)	11.4±4.8 (21)	1.30±0.49 (23)	4.40±1.30 (18)
Normal mice	58.2±18.7 (23)	488.8±262.8 (20)	8.2±3.2 (31)	16.9±6.5 (25)	0.91±0.24 (23)	3.40±1.20 (16)
p value	<0.001	N.S.	<0.01	<0.01	<0.01	N.S.

* ng/ml ** μg/g

All values are expressed as means ± S.D.. In parenthesis, total number of the mice is shown.

Significantly low levels of plasma folate was observed in Rauscher leukemia (RL) compared to the control. As shown in Table 2 anemia, reticulocytosis and increase of nucleated cells were prominent at the stage of the examination (3–4 weeks after virus innoculation). Difference in the folate levels in whole blood was not significant in both groups. Folic acid contents in the liver with RL were lower than those of the control in both free and total folates. Free folic acid content in the spleen with RL was increased significantly though total folate level did not show any significant increase.

2. *Folic acid tolerance test* (Fig. 1):

Plasma folate levels reached its peak after 15 min in both groups. The peak levels in the controls and RL were 138 and 72 ng/ml, respectivelly, this difference being significant. In RL, folic acid level showed significantly lower levels than the control even 30 and 60 min after injecting folate.

3. *The effect of MTX* (Table 2):

No significant changes of the body weight were seen in both groups. Though red blood cells and hematocrit were decreased as the disease progressed, degree of the decrease was more marked in non-treated group than treated group. In the third and fourth weeks of RL virus inoculation, increase of reticulocytes was prevented by giving MTX. In MTX-treated group, the increase of nucleated cells in the peripheral blood was also prevented. The effect of MTX was prominent at the third and fourth weeks of

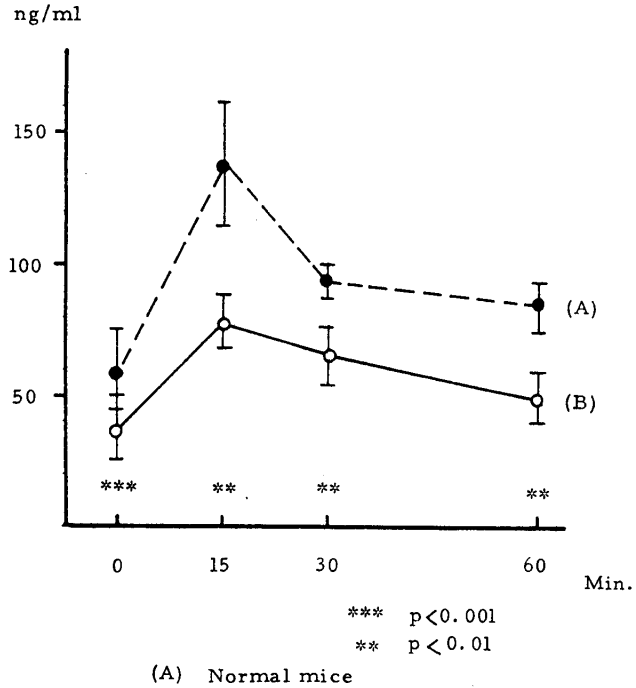

Fig. 1 Folic acid clearance test. Horizontal lines and dots indicate means and S.D., respectivelly. Student-*t* test revealed that folic acid levels were significantly low in Rauscher leukemic mice.

TABLE 2 EFFECTS OF METHOTREXATE ON RAUSCHER LEUKEMIA

		Before	2 Week	3 Week	4 Week
Body Weight	M	23.7±2.9	25.0±2.8	26.2±2.9	26.0±2.6
(g)	C	23.6±2.7	25.8±2.9	25.8±2.9	27.8±2.9
Red Blood Cells	M	1134.6±126.6	983.9±75.3	784.3±89.0***	656.4±123.3**
($\times 10^4$/mm^3)	C	1075.4±108.6	964.0±153.4	593.1±69.4	505.6±111.6
Hematocrit	M	51.0±1.3	48.3±3.3	41.4±4.2***	38.9±6.4***
(%)	C	48.1±1.7	44.7±6.3	32.7±1.4	25.1±4.1
Reticulocytes	M	19.7±7.6	22.7±8.9	76.4±25.2*	110.0±42.0**
(‰)	C	18.3±10.2	43.0±28.1	139.0±56.2	183.2±52.8
Nucleated Cells	M	9306±3295**	7156±2746	20828±4154*	26300±8420***
(/mm^3)	C	4694±1413	9078±4117	49506±32804	54913±17975
Erythroblasts	M	0.22±0.7	1.11±1.5	4.33±2.9**	8.40±5.8***
(%)	C	0	2.33±2.4	11.44±5.8	35.40±12.1

M : Methotrexate treated group (9 mice) C : Control group (9 mice)
* $p<0.05$ ** $p<0.01$ *** $p<0.001$
All values are expressed as means ± S.D.

the course of RL judging from the numbers of erythroblasts.

DISCUSSION

Low serum folate levels in leukemic patients have been well documented in the literature (12, 13, 14, 15). Folic acid deficiency has also been reported in patients with malignant neoplasms (16, 17, 18) and myeloproliferative syndromes (3). Low folate levels in the plasma and the liver revealed in the present study suggest that folic acid deficiency is also present in RL of mice. The clearance test of folic acid showed that disappearance of folic acid from plasma is rapid in RL. This fact coincides well with the observations made in human leukemias and indicates that folic acid metabolism is accelerated in RL. Unexpectedly, an increase of folate in the blood and spleen was observed. This fact rather contradicts the deduction that folic acid deficiency was present in RL. Further study as to what kinds of folic acid derivatives increase in these organs will be needed in order to confirm the above phenomena. As shown in Table 2, the increase of reticulocytes and erythroblasts in the blood was prominent at the third and fourth weeks of RL. According to the previous histological studies of DUNN *et al.* (19), YOKORO *et al.* (20) and IRINO (21), infiltration of erythroblasts in the spleen was also striking at this stage. It is well proved that folic acid contents in immature cells such as reticulocytes and leukemic cells are greater than those in mature cells (22, 23, 24, 25). Therefore, the increase of folic acid in the blood and spleen can be explained by the increase of immature cells in these organs with RL. Then a question arises why folic acid did not increase in the liver. According to the histological studies (19, 20, 21), infiltration of erythroblasts in the liver appeared a week or more after the erythroblastic foci appeared in the spleen. Therefore at 3–4 weeks after virus innoculation, erythroblastic proliferation in the liver was not so extreme as in the spleen. Above all, folic acid content of the liver is about ten times greater than that of the spleen as seen in Table 1. Therefore the total folate content of the liver may be decreased by accelerated metabolism of folate in spite of rather a small increase of folate by the infiltration of immature cells.

MTX is a strong inhibitor of dihydrofolate reductase (26). The enzyme plays a very important role in the biosynthesis of DNA. In RL, as accelerated metabolism of folate is probably due to enhanced biosynthesis of DNA, effect of MTX is expected. The present study revealed that MTX prevents a progress of the leukemia at the third and fourth weeks. The effect of MTX on the survival time of RL was studied by CHIRIGOS *et al.* (27). They indicated that approximately two-fold or greater increase in the median survival time over untreated controls (72 days) were obtained with 0.36 mg/kg

MTX for five days initiated 17 days after the inoculation of the virus. They also suggested that prolongation of survival time was achieved by MTX only after the manifestation of RL and not at an early stage of RL. As MTX can hinder tumor growth by disturbing the biosynthesis of DNA through the deficiency of tetrahydrofolate, MTX seems to be most effective during the period when cell division occurs markedly, *i. e.* at the advanced stage of RL.

As shown in Table 2, progress of anemia is very rapid in the untreated Rauscher mice. Reticulocytosis was also marked. So a question arises whether this anemia is a manifestation of leukemia or that of hemolytic anemia. According to the pathological studies of DUNN *et al.* (19), YOKORO (20) and IRINO (21), infiltration of immature cells in the spleen and the liver indicates that Rauscher disease has a nature of neoplasm. But very rapid progress of anemia cannot be explained only by the infiltration of tumor cells to the hematopoietic organs. Hemolytic process including ineffective erythropoiesis should also be considered. MTX may be effective for the prevention of anemia by reducing the production of immature cells which are susceptible to destruction.

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