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9-Methyl Folate, an Antagonist of Folic Acid : Ist Effect on the Metabolism of Folic Acid in the Rat

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ABSTRACT

The effect of 9-methyl folate on histidine oxidation, the uptake of an injected dose of [^{3}H]folate by the livers and kidneys, the hepatic and blood folate levels were investigated by feeding crude x-methyl folate(XMF) at a level of 5 g per kg diet. 9-Methyl folate is konwn as a major forate antagonist in XMF to produce deficiency signs in rat.

Feeding of XMF decreased histidine oxidation and hepatic folate levels significantly, which showed the function of 9-methyl folate as an antifolate in rats. The hepatic uptake of labeled folate in XMF-fed rats was decreased significantly. These data led to conclude that 9-methyl folate inhibited folate uptake and retention by tissue, especially liver, which could explain the low liver folate levels and the decreased histidine oxidation.

However, only very low level of 9-methyl folate was detected in liver. It suggested that 9-methyl folate may be metabolized very quickly in the liver after uptaken.

KEY WORDS : 9-methyl folate · x-methyl folate · antifolate · folate metabolism.

Introduction

A crude synthetic preparation called "x-methyl folate(XMF)" has previously been shown to function as a folate antagonist for rats and chicks[1)2)]. This crude XMF was synthesized by coupling 2,4, 5-triamino-6-hydroxypyrimidine with p-aminobenzoic acid and 2,3-dibromobutylaldehyde. Since the position of methyl group is uncertain, the crude product was referred as XMF. This product has been found to contain two folate antagonists, 1) 9-methyl folate, present as 5% by weight of the product, which has low activity as a folate antagonist for Streptococcus faecalis, and 2) pyrrofolic acid, a compound present in small amount (0.2%), but having high biological activity for S. faecalis[1)]. However, 9-methyl folate was shown to be a major antifolate to produce deficiency signs in rats and chicks similar to those seen with XMF[3)]. The deficiency was characterized by a slowing of growth, a reduction of hemoglobin, a reduction of white blood cells, and a greater reduction in granulocytes than in the lymphocytes. This syndrome was completely prevented by increasing the level of folate in the diet[1)].

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9-methyl folate had teratogenic effect as the folate analogue was administered to pregnant rats [4)5)].

The activity of the antagonist in depressing cytopoiesis together with the easy reversibility of this activity by folate led to suggestion that this antagonist might be useful in the treatment of leukemia. This experiment deals with the mechanism of antagonistic action of 9-methyl folate in rat by showing its effects on histidine oxidation, the uptake of folate by liver and kidney, and folate levels in various tissues.

Materials and Methods

1. Animals

Female Sprague-Dawley weanling rats(average body weight 50 g) were raised for 5 weeks. Twelve rats were assigned randomly to 3 groups. Folate-free diet based on 20% casein and containing 1% sulfasuxidine was used as a basal diet. The composition of the basal diet is shown in Table

Table 1. Composition of the basal diet

Ingradient	Gram per Kg Diet
Casein	200
Mineral Mix[1]	35
Corn Oil	40
Folate-free Vitamin Mix[2]	10
Choline Chloride	1
Sulfasuxidine	10
Cerelose	704

[1] : Rat mineral mix provided : (ing/kg diet) $CaCO_3$, 6.22 ; $CaHPO_4$, 4.00 ; Na_2HPO_4, 2.00 ; KCl, 7.30 ; $MgSO_4$, 0.46 ; $MnSO_4 \cdot H_20$, 0.05 ; $CuSO_4$, 0.052 ; ferric citrate, 0.025 ; $ZnCO_3$, 0.012 ; KIO_3, 0.0006.

[2] : Vitamin folate-free mix was prepared in glucose and provided : (in mg/kg diet) thiamine · HCl, 15 ; riboflavin, 15 ; pyridoxine · HCl, 15 ; Ca-pantothenate, 50 ; niacine · HCl, 50 ; biotin, 0.2 ; menadione, 10 ; cobalamine, 0.001 ; vitamin A acetate, 15.000 IU ; vitamin D(calciferol), 2000 USP ; D, L-tocopherol acetate, 50

1. Diets and water were fed *ad libitum*. Each group received the following dietary treatment ;

Group A : Basal diet

Group B : Basal diet plus 0.5 mg folate/kg diet

Group C : Basal diet plus 0.5 mg folate/kg diet and 5 g XMF/kg diet which contained 250 mg of 9-methyl folate/kg diet

2. 2-[^{14}C]-Histidine oxidation to $^{14}CO_2$

Histidine oxidation was measured using the procedure of Chan and Stokstad[6)]. The oxidation of 2-[^{14}C]histdine(Amersham, spec. activity, 55.4 Ci/mol) to respiratory $^{14}CO_2$ was measured by intraperitoneal injection of 0.5μCi 2-[^{14}C]histidine per 100g body weight together with 50μmol of unlabeled histidine in 1ml. The rat was placed in a 2500ml metabolic chamber. Air was drawn through the metabolic chamber and into a test tube(25×200mm) containing 30ml of $^{14}CO_2$ trapping fluid(1 : 1, v/v, mixture of monoethanolamine and ethyleneglycolmonomethylether) for 2 hr. A 3ml aliquot was mixed with 10ml of Jeffay-Alvarez scintillation mixture[7)] which gives high counting efficiency(approx. 75%) with this alkaline trapping fluid.

3. Folate uptake and retention by liver and kidney

Folate uptake and retention was measured using the method of Shane et al[8)]. The uptake and retention of folate was determined by intraperitoneal injection of 10μCi purified[3′, 5′, 7, 9, — ^{3}H]folate (20μCi/mmol, Amersham) in 1ml to each rat. after 24hrs of injection the rats were anesthetized by ether and blood was collected by heart puncture and the portions of liver and entire kideys were homogenized with 3 volumes of 2% (w/v) sodium ascorbate buffer(pH 4.3) at room temperature, respectively. The mixture was allo-

wed to be autolyzed for 2 hrs at 37℃ in the dark to hydrolyze the polyglutamate forms of folate to the monoglutamate forms. After autolysis the mixture was boiled at 100℃ for 5 minutes, centifuged at 4000g for 10min, the supernatant collected, and the sample was frozen and stored until analyzed.

The uptake of folate was determined by the percentage of [^{3}H]folate, detected in liver and kidney, of total radioactivity injected. Folic acid concentraton of various tissues was measured by microbiological assay using Lactobacillus casei[9].

4. Analysis of 9-methyl folate by HPLC

The above supernatant of rat liver homogenates prepared by autolysis was centrifuged at 21,000g and the samples were purified on QAE-Sephadex A-25(Phamacia) minicolumn(4×0.7cm) and Sep-pak(Phamacia) and concentrated by evaporation with nitrogen gas. 9-methyl folate was separated by HPLC using Beckman 110A chromatography system. Aliquots(50μl) containing 9-methyl folate were separated on 250×4.6mm(ID) ODS column(Altex). The column was eluted isocratically with 8% dioxane in 50mM citrate buffer, pH 4.2. The flow rate was 1ml/min and 9-methyl folate was monitored at 280nm using UV detector.

Results and Discussions

9-Methyl folate is a major folate analogue in XMF to produce folate deficiency symptoms in rat[1]. In order to study the antagonistic effects of 9-methyl folate, crude XMF was administered to rats in this experimemt.

Addition of XMF to diet produced slight growth retardation in rats(Fig. 1) and the fur became very rough and rare in animals of both group A and C. Most of the rats receiving XMF appeared to have difficulty in eating and tenderness and ulcerative changes in the mouth.

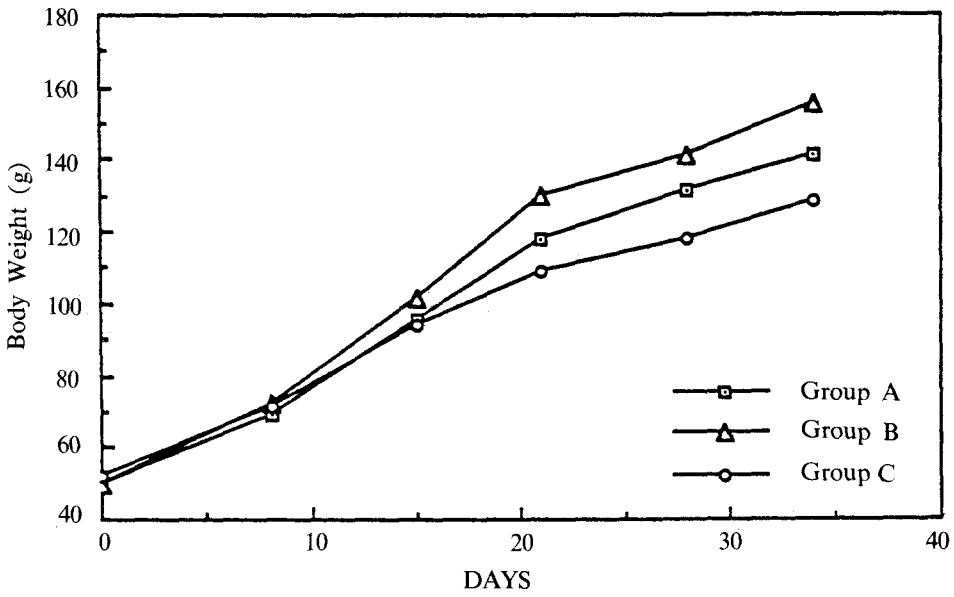

Fig. 1. Body weight changes of rats receiving folate deficient diet(Group A), normal diet supplemented with folate 5mg/kg diet(Group B), and XMF diet supplemented with folate 5mg/kg diet and containing XMF 5g/kg diet.

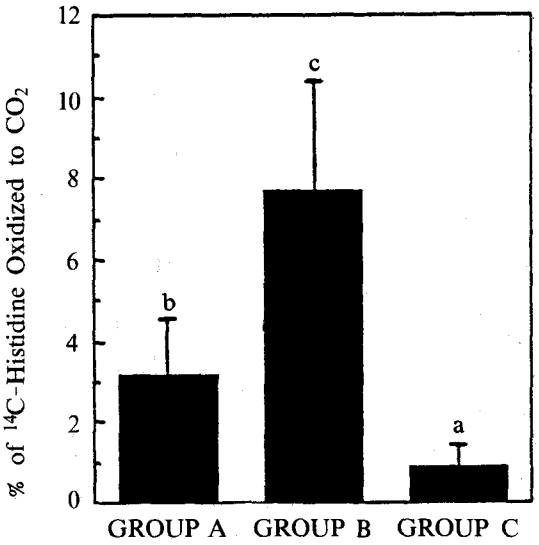

Fig. 2. Histidine oxidation to respiratory $^{14}CO_2$ for 2 hours after an intraperitoneal injection of [2-^{14}C]histidine in rats fed frolate deficient diet(Group A), normal diet(Group B) and XMF diet containing folate and XMF. Data are expressed as Mean± SD. Means with the different letters are significeantly different($P<0.05$) by Duncan's multiple range test.

The most remarkable effect of feeding XMF was found in the rate of histidine oxidation(Fig. 2) and the hepatic uptake of [^{3}H]folate(Fig. 3). The feeding of XMF decreased histidine oxidation to 11% of normal(Group B) and to 27% of folate deficient animals(Group A)(Fig. 2). The severely decreased histidine oxidation in XMF-fed rats is considered to be related with the impaired metabolism of formiminoglutamate by inhibiting tetrahydrofolate formiminotransferase which catalyzes the transfer of the formimino group to tetrahydrofolate.

Hepatic folate levels in folate deficient group and XMF group were 2.3μg and 2.4μg per gram of tissue, respectively, which were only 30% levels of those of control group(Fig. 3). In spite of similar hepatic folate levels in Group A and C, histidine oxidation in Group C was significantly lower than Group A. In this result, the decreased histidine oxidation by the admistration of XMF could not be explained by the low levels of hepatic folate

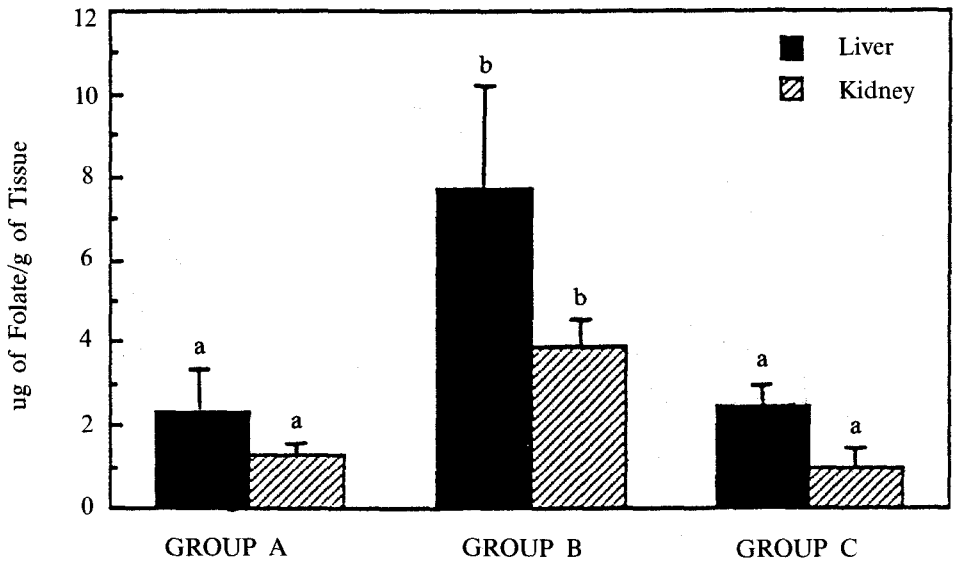

Fig. 3. Hepatic and renal folate levels in rats receiving folate deficient diet(Group A), normal diet(Group B) and XMF diet containing folate and XMF(Group C).
Data are expressed as Mean± SD. Means with the different letters are significantly different($P<0.05$) by Duncan's multiple range test.

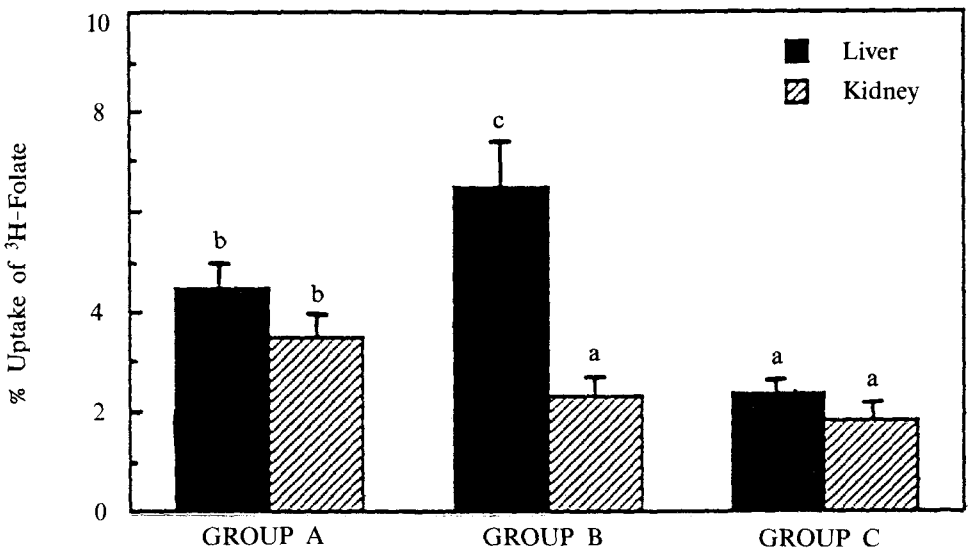

Fig. 4. Uptake of [^{3}H]folate by livers and kidneys of rats receiving folate deficient diet(Group A), normal diet(Group B) and XMF diet containing folate and XMF(Group C).
Data are expressed as Mean± SD. Means with the different letters are significantly different($P<0.05$) by Duncan's multiple range test.

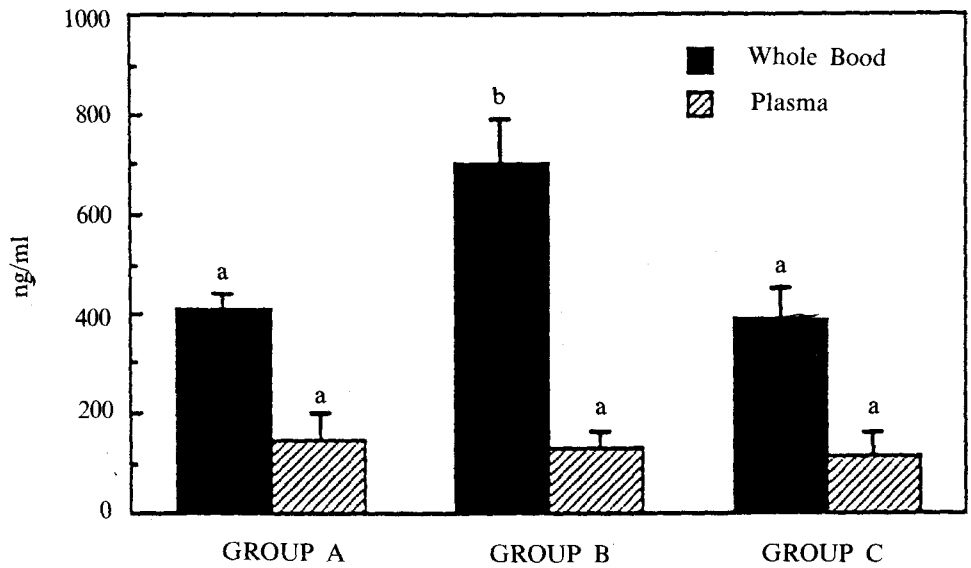

Fig. 5. Folate levels of whole blood and plasma of rats receiving folate deficient diet(Group A), normal diet(Group B) and XMF diet containing folate and XMF(Group C).
Data are expressed Mean± SD. Means with the different letters are significantly different($P<0.05$) by Duncan's multiple range test.

alone. It is possible that 9-methyl folate could change the distribution of the active coenzyme forms of folate in addition to reduce folate levels in liver.

Fig. 4 shows the uptake of labeled folate by rat livers and kidneys after 24 hours of injection. Hepatic uptake of folate, which was measured by injecting [^{3}H] folate intraperitoneally, was decreased in folate deficient group and XMF group. Low folate uptake in the animals fed XMF suggests that the antagonist might compete with folate for transportation into liver cells. It is possible that 9-methyl folate inhibits folylpolyglutamate synthetase[10-13]. As the results the formation of polyglutamate forms of folate and the retention of folate in liver could be decreased[10].

Renal uptake of [^{3}H]folate was significantly lower than hepatic uptake. Renal uptake of folate in XMF-fed rats was similar to normal. The significantly higher renal uptake by folate deficient animals was contrast with the result reported by Shane et al[18]. They observed no significant differance in the renal uptake of [^{3}H]folate of vitamin B12-deficient and supplemented rats.

There was positive correlation between folate levels in liver and whole blood(Fig. 3 & Fig. 5). However, plasma folate level was not affected by either dietary folate levels or folate antagonist.

In this study, 9-methyl folate inhibited the uptake and retention of folate by animal tissue, especially liver, which could explain low liver folate level in XMF-fed rats. Any significant amount of 9-methyl folate, which was analyzed by HPLC, was not found in liver. As the amount of 9-methyl folate was estimated by comparison with internal standard chromatogram, it corresponded to 3ng/50mg of liver, which was approximately 60ng/g of liver. With this level of 9-methyl tolate, this antagonist can not inhibit folate function since 9-methyl folate is known as a weak inhibitor[1)3)] and normally folate is present 3000 to 5000ng per gram of liver. It suggests that 9-methyl folate may be metabolized very quickly in the liver after uptaken. Although the HPLC method used in this experiment could measure 9-methyl folate(ie, oxidized form), if 9-methyl folate was reduced to 9-methyl tetrahydrofolate, the reduced 9-methyl folate could not be analyzed by this HPLC method.

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엽산의 항비타민제인 9-Methyl Folate가 흰쥐의 엽산대사에 미치는 영향

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국문초록

엽산의 항비타민제인 9-methyl folate가 흰 쥐의 엽산 대사에 미치는 영향을 조사하기 위하여, Sprague-dawley 암컷 쥐를 엽산 결핍식이군, 대조군, x-methyl folate 투여군으로 나누어 실험하였다. 9-methyl folate는 실험 동물에게 엽산 결핍증을 유발시키는 x-methyl folate의 성분 가운데 주된 항비타민제이며, 본 실험에서는 식이 1kg당 5g의 x-methyl folate를 첨가하였다.

x-methyl folate를 실험동물에게 먹였을 때 히스티딘의 산화속도와 간장내의 엽산 농도가 크게 저하되었으며, [^{3}H]folate를 복강에 투여한 후 24시간 내에 간장에 보유되는 엽산의 양도 x-methyl folate 투여군에 있어 유의적인 감소를 보였다. 이 실험 결과에서 9-methyl folate는 흰 쥐에 있어 엽산이 간으로 유입되어 보유되는 과정을 저해하므로써 간장 내의 엽산의 양을 저하시키며, 그 결과 히스티딘의 산화속도도 저하된 것으로 보인다.