ABSTRACT

We examined the effects of feeding rats a choline deficient diet, of treating rats with low doses of methotrexate (MTX, 0.1 mg/kg, daily), and of combined choline deficiency and MTX treatment upon the content and distribution of folates in liver. We used a newly devised technique for analysis of folates which utilized affinity chromatography followed by high pressure liquid chromatography. Compared to control rats, total hepatic folate content decreased by 31% in the choline deficient rats, by 48% in the MTX treated rats, and by 60% in rats which were both choline deficient and treated with MTX. In extracts of livers from control rats, folates were present predominantly as penta (35%) and hexaglutamyl (57%) derivatives. The pteridine ring structure distribution of these folates was as follows: 48% 5-methyltetrahydrofolate, 14% formylated tetrahydrofolate, and 39% tetrahydrofolate. In choline deficient animals, there was a decrease in the relative concentration of pentaglutamyl folates and an increase in the relative concentration of heptaglutamyl folates. In livers from MTX treated animals, MTX-polyglutamates with 2–5 glutamate residues accumulated. The consequences of MTX treatment were: a) an elongation of the glutamate chains of the folates as the proportion of hepta- and octaglutamyl derivatives was increased relative to penta- and hexaglutamyl folates; b) the occurrence of unredced folic acid; c) a decrease in the relative concentration of 5-methyltetrahydrofolate and an increase in the relative concentration of formylated tetrahydrofolate, and d) no change in the relative concentrations of tetrahydrofolate. In livers from animals that were both choline deficient and treated with MTX, the tetrahydrofolate concentrations were 50% of control while formylated tetrahydrofolate concentrations increased 3-fold. These data are discussed from the standpoint of the current understanding of mechanisms that regulate the elongation of the glutamic acid chains of folates and those that regulate folate dependent synthesis and utilization of one carbon unit.

INTRODUCTION

Folate and choline metabolism are interrelated, and perturbations in one pathway affect the other. The transmethylation of homocysteine by 5-methyltetrahydrofolate is a most important folate dependent reaction because the product, methionine, is the precursor of S-adenosylmethionine (1). Methionine biosynthesis via this pathway cannot meet all demands for AdoMet2 and, therefore, dietary sources of labile methyl groups (methionine and choline) are required (2). Choline, after conversion to betaine, can act as the methyl donor for homocysteine by 5-methyltetrahydrofolate is a most important reaction catalyzed by betaine transmethylation, a reaction which is similar to those seen in choline deficiency (13). Animals on low doses of MTX have diminished hepatic folate levels of AdoMet, methionine, and betaine (13, 16–18). This effect of MTX is most likely to be due to impairment of de novo synthesis of methionine due to alterations in folate metabolism, including diminished capacity of the liver to retain folate (19, 20). MTX is widely used as a chemotherapeutic agent (21–23) and, at low doses, for the treatment of psoriasis (24), rheumatoid arthritis (25, 26), and certain liver disorders (27–29). In chemotherapy, MTX is often administered in combination with other agents, many of which are carcinogens (15). Diminished tissue stores of choline are seen in humans fed i.v. (30); parenteral nutrition is often used in cancer patients. In rats, choline deficiency and treatment with MTX significantly increase the incidence of procarbazine induced mammary tumors (15). It is possible that lipotrope or folate deficiency, caused by choline deficiency or MTX treatment, might increase the carcinogenicity of chemotherapeutic agents in humans.

The present study was undertaken to determine the effects of choline deficiency and methotrexate treatment (alone and in combination) on hepatic folate distribution. Hepatic folate dis-
tribution was determined by using a new method which combines affinity chromatography with high pressure liquid chromatography (31, 32). One advantage of this method is that it provides information on the pteridine ring distribution as well as changes in the glutamate chain lengths.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats (approximately 200 g body weight; Charles River Breeding Laboratories, Wilmington, MA) were housed in suspended stainless steel wire cages in a climate controlled room (24°C), and were exposed to light from 6 a.m. to 6 p.m. daily. Animals were fed the control diet (ICN Nutritional Biochemicals custom control diet: 10% casein, 10% α-soy protein, 20%lard, 56% sucrose, 4% salt mix W, ICN vitamin mix with choline; the diet contained final content of 0.2% choline, 0.27% cystine, and 0.35% methionine) for 1 week prior to use in order to acclimatize them to a semisynthetic diet.

Rats were randomly assigned to 4 treatment groups (n = 5/group) and pair fed a control or choline deficient diet (same as the control diet except that we used ICN vitamin mix without choline; it contained a final content of 0.002% choline) for 2 weeks. Five animals on each diet were treated daily for 2 weeks with MTX (0.1 mg/kg body weight administered i.p. at midday; Behring Diagnostics, La Jolla, CA). On day 15, the rats were anesthetized with diethylether and samples of liver were removed, immediately weighed, and placed in 5 volumes of boiling extraction solution (10 mM 2-mercaptoethanol and 2% sodium ascorbate in 0.1 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane buffer at pH 7.8). After 15 min in the boiling bath the extraction solution was cooled on ice, the mixture was homogenized, and the supernatant fraction was collected after centrifugation at 20,000 × g for 15 min (31).

Analysis of folate was conducted by using a new method (31) which combines affinity chromatography with the use of immobilized milk folate binding protein with HPLC for direct determination of folate distribution. An aliquot of the supernatant fraction (with an estimated total folate plus MTX content not exceeding 15 nmol) was mixed with a trace amount of [3H]folic acid (5 μCi, 40 Ci/mmol; Amersham, Arlington Heights, IL) and applied to a folate binding protein-Sepharose (affinity) column (32). The column (1 ml bed volume) was washed sequentially with 15 ml 1 M potassium phosphate buffer, pH 7.0, 15 ml water, and then eluted with 4 × 1 ml 0.02 M trifluoroacetic acid containing 0.01 M dithioerythritol. The acidic fractions were promptly neutralized with 1 M piparazine. Aliquots of these fractions were used to determine 3H content; a recovery of 90% or more of applied radioactivity was taken as an indication that the affinity column was not overloaded. The remainder of the neutralized fractions were combined and an aliquot of 0.9 ml was used for analysis of folate distribution by ion pair liquid chromatography, using diode array detection (31). Briefly, the sample was injected onto a C8 HPLC column (Econosphere, 5 μm, 4.6 × 100 mm; Alltech, Deerfield, IL) which had been equilibrated with 5% tetrahydroammonium phosphate, 25 mM NaCl, 5 mM dithioerythritol, and 10% acetonitrile. After sample application, folates were eluted from the column by using a linear gradient (10–65%) of acetonitrile in the same equilibration solution. In this chromatographic system, folates elute in the order of increasing number of glutamate residues. Those with the same number of residues elute as a separate cluster arranged in three groups. Group 1 consists of 10-formyltetrahydrofolates (10-formyl-H4PteGlu; F1), tetrahydrofolates (H4PteGlu, T4), and dihydrofolates (H4PteGlu, D4). Group 2 consists of 5-formyltetrahydrofolates (5-formyl-H4PteGlu; L5), and folates (PteGlu, P); the diode array detection system permitted the monitoring of the column effluent at 280 nm for determination of folate activity and information on the number of glutamate residues of any eluting folate. A final identification of the pteridine structure of the eluting folate was made on the basis of the UV absorption values at 350 and 258 nm. Absorption values at 350 nm were identified between T4 and F1. Integrated peak areas were used for estimation of folate concentrations of the individual peaks and the sum of these integrated areas were used to determine total folate content of the sample. A mixture of PteGlu,1-7 (1 nmol each) standards was run through the column after chromatography of 2–3 samples. These runs served to determine the outer boundaries of the respective clusters (32).

The monoglutamyl-MTX (X1) and its polyglutamate cogeners (X2–X4) were identified on the basis of retention time determined with authentic standards and spectral properties. Fig. 1 shows a typical chromatogram of MTX standards. When the affinity column was not overloaded with other folates, retention of MTX was greater than 90%.

When data are expressed as nmol/g folate liver, MTX concentrations were not included. Data were analyzed for statistical significance by using analysis of variance and appropriate critical difference tests with Tukey’s honestly significant difference method.

**RESULTS**

All animals gained similar amounts of weight during the 2 weeks of treatment (77–84 g). There were no significant differences in hepatic protein concentrations (142–150 mg/g liver). As compared with controls, rats fed the choline deficient diet had 3-fold more hepatic triacylglycerol and diminished hepatic concentrations of choline (≤50% of control), betaine (30% of control), methionine (80% of control), and AdoMet (60% of control). Liver S-adenosylhomocysteine concentration increased by 25%. Methotrexate treatment alone was associated with decreased concentrations of hepatic betaine (55% of control), AdoMet (75% of control), and methionine (88% of control). Combined treatment with MTX and choline deficiency further diminished hepatic AdoMet concentrations (33% control) and increased S-adenosylhomocysteine (to 150% of control). A full report of these changes has been published elsewhere (13).

Fig. 2 shows ion pair HPLC of representative purified liver extracts from the four treatment groups. The two major clusters in Fig. 2 are typical of rat liver folates (32) and consist of reduced penta- and hexaglutamyl derivatives. The first peak in each cluster contains 10-formyl-H4PteGlu,(F1) and unsubstituted H4PteGlu,(T1). The second peak, which is a minor constituent, represents 5-formyl-H4PteGlu,(L5), whereas the last peak consists of 5-methyl-H4PteGlu,(M4). In addition to these two clusters, the liver extracts contained traces of heptaglutamyl folate derivatives (T2 and M2) and of 5-methyl-H4PteGlu,(M1).
Except for minor differences, which will be dealt with later, the pattern of hepatic folate distribution in choline deficient animals (Fig. 2B) was similar to that from the livers of control animals.

Chromatography of the purified liver extracts from the methotrexate treated animals (Fig. 2, C and D) yielded additional peaks (X1-X5) which represent MTX polyglutamates containing (total) 2-5 glutamate residues. These samples contained larger proportions of heptaglutamyl and octaglutamyl folates and lesser proportions of pentaglutamyl folates. In addition, in the region of monoglutamyl folates, unmodified PteGlu1(P1) was present and there was no 5-methyl-H4PteGlu1(M1).

Total folate in the liver was decreased by 31% in choline deficient animals (P < 0.05), by 48% in MTX treated animals (P < 0.01), and by 60% in animals that were both choline deficient and treated with MTX (P < 0.01) (Fig. 3). Fig. 4 shows the distribution of MTX polyglutamates in livers of MTX treated animals. In both the choline sufficient and the choline deficient groups, polyglutamation did not extend beyond the pentaglutamyl level. The degree of polyglutamation was increased in the choline deficient rats.

Folate distribution (Fig. 5) was similar in the control and choline deficient rats. However, the portion of total folates present as the H4PteGlu5(T5) cogenor was decreased (P < 0.05). The portion present as T7 tended to increase; however this increase was not statistically significant. Distribution based upon the pteridine ring structure was similar in the livers from choline deficient and control rats (Fig. 6).

MTX treatment was associated with the elongation of the glutamic acid chains of folates (Fig. 5). There was a decrease in the concentration of pentaglutamyl (folate) derivatives and an increase in the relative concentration of heptaglutamyl folates. This effect was even more pronounced in livers from the choline deficient/MTX treated rats, where pentaglutamyl folates decreased to less than 10% of the total, hexaglutamyl folates decreased to 31% of the total, while heptaglutamyl folates increased 38 and 20% of total folates, respectively (P < 0.05) (Fig. 5). MTX treatment was associated with the doubling in the relative concentrations of formylated tetrahydrofolates (F4 + F6), while a relative concentration of 5-methyltetrahydrofolates decreased by 26% when compared to control (P < 0.01) (Fig. 6). The relative concentration of tetrahydrofolate derivatives were unchanged from control. In livers from the choline deficient/MTX treated rats, the relative concentration of 5-methyltetrahydrofolates in the liver decreased by 26%, tetrahydrofolate relative concentrations decreased by 50%, while the formylated tetrahydrofolate relative concentrations tripled (P < 0.01) (Fig. 6).

DISCUSSION

Numerous reactions use folate containing coenzymes. Regulation of folate dependent metabolism occurs, in part, at the level of these coenzymes. The coenzyme which mediates one reaction may inhibit a second folate dependent reaction and coenzyme activity/inhibitor status may depend upon the chain.
EFFECTS OF CHOLINE AND METHOTREXATE ON HEPATIC FOLATE

The capacity for analyzing the distribution of the various folates within tissues is of importance, as it provides insights into the changes in tissue specific folate dependent metabolic activity in response to drugs and nutrients. This concept has not been widely used because of difficulties in resolving the many forms of folates that may exist within the same tissue. In fact, most studies on tissue folate distribution used methods which included steps to reduce the number of derivatives to a more manageable number (35, 36). The method that was developed in our laboratory (31, 32) and used in the present study is both simple and rapid and provides information on the content and distribution of folates with emphasis on both ends of the folate molecule. In fact, the chromatographic analyses of the purified liver folates from the MTX treated animals are the first of their kind. Previous studies on the effect of MTX on folate distribution in the cell relied on analysis of radioactivity distribution following the addition of radiolabeled folate and allowing time for equilibration with endogenous folate (35).

We have shown that choline deficiency and MTX treatment, alone or in combination, caused both quantitative and qualitative changes in hepatic folates. These changes are likely to represent changes in the content and distribution of folate in hepatocytes and are not due to fatty infiltration of the liver (hepatic protein concentrations were not significantly different in the various treatment groups). MTX treatment was associated with a) the accumulation of MTX polyglutamates; b) the appearance of unmodified PteGlu; c) elongation of the glutamate chains; and d) a decrease in the proportion of 5-methylTHF, an increase in the proportion of formylated THF, and no change in the proportion of THF.

Under normal conditions, PteGlu, which was the sole source of folate in the experimental diet used in this study, is reduced and methylated in the course of transport across the intestinal wall (37) and/or transport into hepatocytes (38). This reduction has two important functions. It provides coenzymes for those enzymes which are involved in one carbon metabolism as well as the substrate (in the form of H4PteGlu) for the synthesis of folate polyglutamates (39). MTX inhibits the reduction of PteGlu to H4PteGlu and, as a consequence, both the supplies of folate coenzymes and of the H4PteGlu substrate for the conversion to polyglutamyl derivatives are impaired. The unmodified PteGlu, which is poorly polyglutamated, leaks off the cell (40).

MTX is also an inhibitor of the transport of PteGlu across the intestine and into hepatocytes (41, 42) and this would contribute further to the impairment of folate supply to the tissue. In fact there are indications that MTX affects the assimilation into the body folate pool of reduced folates as well (19, 43, 44). These multiple sites of inhibition strongly suggest that in the course of MTX treatment little, if any, of the PteGlu from the diet was assimilated into the liver folate pool. This possibility is in line with the observed accumulation in the liver of large concentrations of MTX polyglutamates which are accompanied by the occurrence of unmodified PteGlu.

Elongation of the glutamate chains of folates in the liver is the function of both the pteridine ring structure and the number of glutamic acid residues attached (37). With tetrahydrofolates (the most effective substrates), elongation is most rapid when the total glutamate residues number 5 or less. Beyond H4PteGlu, additional glutamates are attached at much lower rates due to a sharp drop in the affinity of polyglutamate synthase. Folate depletion which accompanies MTX treatment...
may result in a slower turnover of the residual folate and hence, a greater than normal residence time to allow the synthesis of heptaa- and octaglutamyl folates. Elongation of the glutamate chain length of folates was also reported by Cassady et al. in livers from folate deficient rats (45) and in a number of other conditions including acute alcohol intake (46). In some of these instances chain elongation may be explained on the basis of increased residence time of the folate within the liver (47). In other cases this relationship is not obvious.

MTX and its polyglutamate derivatives are strong inhibitors of aminomidazolcarboxamide ribonucleotide transformylase (33). This inhibition was thought to be responsible for the inhibition of purine synthesis in MCF-7 breast cancer cells (48) and could explain the data from the present study which showed an increase in the relative concentration of formylated THF in livers of rats treated with MTX. An alternative, but not mutually exclusive, interpretation is related to the observed decrease in hepatic methionine and AdoMet concentrations in MTX treated rats. Undoubtedly depletion of hepatic folate supply will impair the de novo synthesis of methionine. However, the observation that MTX treatment is associated with a sharp decline in 5-methyl-THF concentrations point to the possibility of impairment in the synthesis of this folate. This effect of MTX on 5-methyl-THF concentration in rat liver was also reported earlier by Barak et al. (49) and in cell cultures by Kesavan et al. (50) and by Allegra et al. (48). Methylenyl-THF reductase, which catalyzes the synthesis of 5-methyl-THF, was strongly inhibited by dihydrofolate polyglutamates (34), and it is possible that this inhibition is exerted also by MTX and its polyglutamate derivatives as reported by Chabner et al. (51).

The above interpretation, that MTX is an antagonist of methionine synthesis and is an inhibitor of the synthesis of 5-methyl-THF is also consistent with the changes seen in hepatic folate distribution in rats which were both choline deficient and treated with MTX. These changes cannot be explained on the basis of additive effects of individual contributions of choline deficiency and MTX treatment. Choline deficiency, which alone had no effect on hepatic folate distribution, in combination with MTX caused a net increase in the accumulation of formylated THF and a sharp decrease in the concentration of THF. These changes in folate distribution are the same as those observed by Horne et al. in rats fed a restricted choline/methionine diet (14).

The increasing use of low doses of MTX for long periods of time to treat a variety of chronic illnesses raises a number of important questions regarding potential benefits and side effects that this treatment causes. In humans, MTX treatment appears also to result in the decrease of folate body pool (43, 44). The present study provides basis of understanding some of the complex interrelationships between choline, folate, and MTX metabolism.

REFERENCES

EFFECTS OF CHOLINE AND METHOTREXATE ON HEPATIC FOLATE


Effects of Choline Deficiency and Methotrexate Treatment upon Liver Folate Content and Distribution

Jacob Selhub, Elias Seyoum, Elizabeth A. Pomfret, et al.


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