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INTERACTION OF METHOTREXATE WITH LIPOTROPIC FACTORS IN RAT LIVER*

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Abstract—Methotrexate (MTX), a drug used in cancer chemotherapy and in the treatment of psoriasis, is known to impair DNA and RNA synthesis by antagonizing folate metabolism. A major side-effect of this drug is the formation of a fatty liver and eventual cirrhosis of the liver. Since levels of certain lipotropic substances in the liver are also synthesized *de novo* by way of folate-dependent 1-carbon metabolism, this study was conducted to determine if the side-effects of MTX may be a consequence of interference with 1-carbon metabolism in the liver. This study investigated the effect of MTX on hepatic and serum lipid content when various lipotropes were administered to rats fed a diet deficient in methionine, choline and vitamin B_{12}. Results demonstrate that MTX can potentiate a choline-deficient fatty liver and block the lipotropic effect of vitamin B_{12}. They also show that methionine and choline, products of 1-carbon metabolism beyond folate involvement, overcame the detrimental effects of MTX. This suggests that MTX interferes with hepatic methyl group genesis and hence blocks the *de novo* synthesis of methionine and choline.

An antimitotic drug, 4-amino-10-methylpteroylglutamic acid or methotrexate (MTX), used extensively in the treatment of cancer and psoriasis, is an effective inhibitor of dihydrofolate reductase (EC 1.5.1.4.) [1–7]. Inhibition of this enzyme results in impaired production of tetrahydrofolic acid, transfer of 1-carbon units and production of purine and pyrimidine bases. This leads to the ultimate decreased synthesis of DNA and RNA in the cell. The inhibition of nucleic acid synthesis by MTX appears to be the most significant effect of this drug in damaging rapidly growing cells and could explain its use as an effective agent in cancer chemotherapy [8].

One major disadvantage in using MTX in the treatment of cancer and psoriasis has been that this drug is a potent hepatotoxin [9–12]. Patients treated for cancer frequently develop severe abnormalities in liver function tests and portal cirrhosis [11–13]. Even at low dosages employed in psoriatic patients, liver damage and even cirrhosis have occurred [14–16]. These severe side-effects of MTX have limited its general use and have increased the need for greater precautions whenever this drug is administered.

In addition to their role in nucleic acid synthesis, tetrahydrofolic acid coenzymes also function in 1-carbon transfers which are essential for methyl group generation and subsequent methionine synthesis in the liver. These methyl transfers are essential for the *de novo* genesis of the key lipotropic substance, choline. Since this process may be important in man [17], it is possible then that the side-effects of MTX occurring in the liver may result from the interference of this drug with the generation of methyl groups for choline synthesis. This study sought to test this hypothesis by measuring the effect of MTX administration on hepatic fatty infiltration in rats fed a lipotropic-deficient diet and the effects of MTX administration on similarly fed animals when the diet was individually supplemented with the lipotropes, methionine, choline or vitamin B_{12}.

MATERIALS AND METHODS

Experiment 1

This experiment was designed to study the effect of MTX on hepatic and serum lipid content when various lipotropes were administered to rats fed a basal lipotropic-deficient diet. Male, Sprague–Dawley rats (80–120 g) were maintained on normal laboratory rat pellets for 7 days after their arrival from the animal breeders. After this period, the rats were divided into eight groups and fed *ad lib.* for 10 days on the basal lipotropic-deficient diet of Iseri *et al.* [18]. This diet was deficient in choline, methionine and vitamin B_{12} but contained adequate supplements of folate and homocysteine. During the experimental time period, the animals received injections or dietary supplements as outlined in Table 1. The methotrexate-treated animals were injected intraperitoneally with 0·1 mg/kg daily for the 10-day period. This dose was about three times that used in the treatment of psoriatic patients and was found to be sub-lethal in rats in initial pilot studies. Those rats receiving vitamin B_{12} were given daily doses of 140 μg/kg, i.p. Those animals not receiving injections of MTX or vitamin B_{12} or both received daily sham injections of saline in equivalent volumes. Supplements of methionine and choline when given were added to the diet at levels of 0·5 and 0·3 per cent respectively. On the killing date, the non-fasted animals were placed under ether anesthesia, exsanguinated to obtain blood samples and the livers removed for lipid analyses. Serum was prepared from the blood samples and the chylomicrons were removed by centrifuging serum at 9500 *g* for 30 min in a 40·3 rotor using a model L

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Table 1. Diet, dietary supplementations and injections given to rats

Group	No. of rats	Diet	MTX
1	5	Basal-deficient	−
2	4	Basal-deficient + choline	−
3	5	Basal-deficient + choline	+
4	6	Basal-deficient + vitamin B_{12}	−
5	7	Basal-deficient + vitamin B_{12}	+
6	4	Basal-deficient + methionine	−
7	3	Basal-deficient + methionine + vitamin B_{12}	−
8	5	Basal-deficient + methionine + vitamin B^{12}	+

Beckman–Spinco ultracentrifuge [19]. The infranatant solution was used for lipid analyses. Total lipids were extracted by homogenization with chloroform–methanol (2:1, v/v) according to Folch *et al.* [20]. Free cholesterol and cholesterol esters were determined by the method of Searcy and Berquist [21]; triglycerides were measured by the method of Soloni [22] and phospholipids' levels were estimated by the method of Bartlett [23].

Experiment 2

This experiment was conducted to determine if MTX administration would potentiate the fatty liver and the hypolipemia induced by a choline deficiency. Male, Sprague–Dawley rats (100–150 g) were divided into two groups. The experimental group was given daily injections of methotrexate (0·1 mg/kg, i.p.) and the control group sham injections of saline for a period of 7 days. After this time both groups were fed the choline-deficient diet of Iseri *et al.* [18] and the injections of MTX and saline continued on a daily basis. Pre-injection of MTX was deemed necessary to allow the drug enough time to function before the imposition of the choline deficiency. One-half of the control and experimental animals were killed after 2 days and the remainder of the animals were killed after 5 days and liver and blood were prepared for lipid analysis. The 2-day and 5-day periods were chosen in order to test the effect of the drug in an early choline deficiency when the liver is in the initial stages of fatty infiltration.

RESULTS

During the 10 days of Experiment 1, the animals showed a 40–45 *g* increase in body weight with no differences in weight among the rats in the various eight groups. Table 2 portrays the hepatic lipid pattern in rats after 10 days on MTX and various lipotropic regimens. Hepatic triglyceride and cholesterol esters were highly elevated in the rats fed the basal lipotropic-deficient diet. Supplements of choline and methionine to the basal diet reduced these levels by 90 per cent or more. Addition of B_{12} to the basal diet reduced triglyceride and cholesterol ester levels by about 50 per cent.

Table 2 also shows that MTX had no significant effect on the hepatic triglyceride and cholesterol ester levels in the animals fed both the choline- and methionine-supplemented diets. However, MTX

Table 2. Hepatic lipids in methotrexate-treated rats

		Triglyceride				Cholesterol esters	
Group	Supplement(s)	(μmoles/ total liver)*	P	(mg/g)*	P	(μmoles/ total liver)*	P
1	None	3390 ± 899		313 ± 50		559 ± 47	
2	Choline (0·3%)	139 ± 42	<0·001†	17·4 ± 2·1	<0·001†	37·8 ± 8·2	<0·001†
3	Choline (0·3%) +MTX	99 ± 17	<0·001† <0·1‡	11·7 ± 1·7	<0·001† <0·01‡	31·2 ± 4·9	<0·001† NS§
4	B_{12}	2256 ± 744	<0·01†	186 ± 34	<0·001†	264 ± 80	<0·001†
5	B_{12} + MTX	3667 ± 573	NS‖ <0·01¶	289 ± 32	NS‖ <0·001¶	372 ± 53	<0·001† <0·02¶
6	Methionine (0·5%)	347 ± 62	<0·001†	37·2 ± 8·3	<0·001†	76·3 ± 16·2	<0·001†
7	Methionine (0·5%) + B_{12}	283 ± 72	<0·001† NS**	31·7 ± 3·8	<0·001† NS**	88·0 ± 18·7	<0·001† NS**
8	Methionine (0·5%) + B_{12} +MTX	307 ± 126	<0·001† NS**	33·1 ± 10·6	<0·001† NS**	89·2 ± 44·3	<0·001† NS**

* Mean values ± S.D.
† Compared to Group 1.
‡ Compared to Group 2.
§ NS = not significant compared to Group 2.
‖ NS compared to Group 1.
¶ Compared to Group 4.
** NS compared to Group 6.

Table 3. Serum lipids in methotrexate-treated rats

Group	Supplement(s)	Triglyceride*	P	Total cholesterol*	P	Phospholipids*	P
1	Basal	0·11 ± 0·04		1·32 ± 0·18		0·87 ± 0·13	
2	Choline	0·47 ± 0·22	<0·01†	2·50 ± 0·35	<0·001†	1·40 ± 0·07	<0·001†
3	Choline (0·3%) +MTX	0·43 ± 0·14	<0·002† NS‡	2·32 ± 0·38	<0·001† NS‡	1·25 ± 0·13	<0·002† NS‡
4	B_{12}	0·21 ± 0·04	<0·01†	1·57 ± 0·08	<0·05†	0·89 ± 0·09	NS§
5	B_{12} + MTX	0·16 ± 0·08	NS§ NS‖	1·40 ± 0·16	NS§ <0·05¶	0·83 ± 0·07	NS§ NS‖
6	Methionine (0·5%)	0·48 ± 0·08	<0·001†	2·58 ± 0·55	<0·002†	1·42 ± 0·26	<0·01†
7	Methionine (0·5%) + B_{12}	0·36 ± 0·08	<0·002† NS**	2·70 ± 0·20	<0·001† NS**	1·19 ± 0·22	<0·05† NS**
8	Methionine (0·5%) + B_{12} +MTX	0·36 ± 0·18	<0·02† NS**	2·58 ± 0·15	<0·001† NS**	1·44 ± 0·25	<0·002† NS**

* Mean values in μmoles/ml ± S.D.
† Compared to Group 1.
‡ NS = not significant compared to Group 2.
§ NS compared to Group 1.
‖ NS compared to Group 4.
¶ Compared to Group 4.
** NS compared to Group 6.

administration to the B_{12}-supplemented group returned the triglycerides to the high level seen in the rats of the basal group and increased the cholesterol esters to the levels approaching those of the basal group. Thus, MTX blocked the lipotropic effect of B_{12}. Hepatic phospholipids remained constant throughout the various diet and drug manipulations. Free cholesterol was only slightly elevated in the basal group, and no differences were observed in the other seven groups.

Table 3 manifests the serum lipid patterns of Experiment 1, and they reflect the status of hepatic lipid transport during this study. This table shows that after 10 days of feeding the basal lipotrope-deficient diet serum lipids are significantly lower than those in the choline- and methionine-supplemented animals. Vitamin B_{12} supplementation also increased serum lipid levels when compared to the basal group, but not to the extent seen in the methionine- and choline-supplemented groups. As was the case with the hepatic lipids, MTX administration to the rats in the methionine- and choline-supplemented groups had no effect on serum lipids. On the other hand, MTX was effective in lowering serum cholesterol levels when administered to the rats in the B_{12}-supplemented groups and lowered serum triglyceride levels near those of the basal group. Thus, in regard to serum lipids, MTX also seems to antagonize the lipotropic action of B_{12}.

Table 4 shows that in an early choline deficiency (2 day) MTX potentiates the accumulation of triglyceride and cholesterol esters in the liver. This potentiation effect, however, appears to be lost or masked in a later choline deficiency (5 day) when the accumulation of triglyceride and cholesterol esters is six to seven times the 2-day levels. Free cholesterol and phospholipid did not appear to be affected by MTX in either the 2-day or 5-day choline deficiency. Table 5 shows the effect of MTX on serum lipids of choline-deficient rats. MTX lowered the serum triglyceride and phospholipid levels both in the 2-day and 5-day choline-deficient rats. Cholesterol levels were unaffected by MTX treatment. These results indicate that MTX potentiates the hepatic steatosis in an early choline deficiency and that it also potentiates the hypolipemic condition of a choline deficiency.

DISCUSSION

One-carbon transfer reactions mediated by tetrahydrofolate (THF) coenzymes are important to the total metabolic processes in the organism. For example, 1-carbon metabolism is involved in the methylation of purines and pyrimidines in the biosynthesis of DNA and RNA and also in the methylation of tRNA. These processes are essential to growing cells. The drug methotrexate (MTX) has been shown to be a folate antagonist by its ability to inhibit generation of tetrahydrofolate coenzymes [24] and hence is effective in cancer chemotherapy and in the treatment of psoriasis. A major disadvantage of MTX administration is that it is hepatotoxic, producing fatty metamorphosis in the liver and portal cirrhosis even when small doses are given [9–16].

Another important function of the THF coenzymes is the *de novo* generation of methyl groups for hepatic methionine and choline biosynthesis via the methylation of homocysteine [25, 26]. In this process, the presence of vitamin B_{12} is known to be required [18, 27, 28]. It is possible that the adverse side-effects of MTX in producing liver injury may be due in part to an inhibition of the biosynthesis of the lipotropic substances, methionine and choline, through its interference with hepatic 1-carbon metabolism.

When a rat is fed a choline-deficient diet, which is also low in methionine, the main source of choline for the liver must be the sequential methylation pathway [26]. THF coenzymes are responsible for the generation of the methyl groups needed for this pathway. If this 1-carbon transfer system is blocked by MTX, it is feasible that the impaired lipid transport

Table 4. Effect of methotrexate on hepatic lipids in rats fed a choline-deficient (CD) diet

Type	No.	Triglycerides* (μmoles/total liver)	Triglycerides* (mg/g wet wt)	Free cholesterol* (μmoles/total liver)	Cholesterol esters* (μmoles/total liver)	Phospholipids* (μmoles/total liver)
CD controls (2-day)	5	235 ± 95	35·3 ± 12·2	26·7 ± 8·1	67·4 ± 10·3	182 ± 35
CD-MTX-treated (2-day)	6	366 ± 108†	50·7 ± 14·0‡	24·9 ± 5·6§	96·5 ± 26·9†	200 ± 42§
CD controls (5-day)	6	1718 ± 341	186 ± 26	30·7 ± 6·6	392 ± 92	200 ± 43
CD-MTX-treated (5-day)	5	1674 ± 451§	173 ± 32§	28·6 ± 4·7§	389 ± 132§	212 ± 32§

* Mean values ± S.D.
† $P < 0.05$ when compared to control values.
§ Not significant when compared to control values.
‡ $P < 0.1$ when compared to control values.

from the liver would be potentiated and the effects of the choline deficiency would be magnified. The increased accumulation of triglyceride and cholesterol esters in the MTX-treated animals as shown in Table 4 as well as the increase in the severity of the hypolipemia, demonstrates that the drug does potentiate an early choline deficiency and lends support to the hypothesis that MTX may indeed impair the *de novo* synthesis of methionine and choline.

Vitamin B_{12} is required for the enzymatic reaction in which 5-methyltetrahydrofolate transfers its methyl group to homocysteine forming methionine and hence choline [18]. The data in Table 2 which show that MTX negates the effect of vitamin B_{12} in reducing hepatic fat accumulation further the hypothesis above. The data in the same table show that the lipotropic action of methionine and choline (products of 1-carbon metabolism beyond folate involvement) is not inhibited by MTX. This suggests that the side-effects of this drug may be involved in the interference with the synthesis of these key lipotropes and not in the degradation of these substances.

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REFERENCES

1. J. F. Holland, *Clin. Pharmac. Ther.* **2,** 374 (1961).
2. W. M. Hryniuk and J. R. Bertino, *J. clin. Invest.* **48,** 2140 (1969).
3. J. R. Bertino, M. Levitt, J. L. McCullough and B. Chabner, *Ann. N.Y. Acad. Sci.* **186,** 486 (1971).
4. D. A. Karnofsky and B. D. Clarkson, *A. Rev. Pharmac.* **3,** 357 (1963).
5. R. B. Rees, J. H. Bennet and H. J. Mailbach, *Archs Derm.* **95,** 2 (1967).

Table 5. Effect of methotrexate on serum lipids in rats fed a choline-deficient (CD) diet

Type	No.	Triglyceride* (μmoles/ml)	Total cholesterol* (μmoles/ml)	Phospholipids* (μmoles/ml)
CD controls (2-day)	5	0·47 ± 0·17	2·84 ± 0·23	1·44 ± 0·21
CD-MTX-treated (2-day)	6	0·26 ± 0·05†	2·52 ± 0·37‡	1·13 ± 0·18§
CD controls (5-day)	6	0·30 ± 0·08	1·72 ± 0·20	0·99 ± 0·16
CD-MTX-treated (5-day)	5	0·17 ± 0·02§	1·71 ± 0·38‡	0·83 ± 0·14‖

* Mean values in μmoles/ml ± S.D.
† $P < 0.02$ when compared to control values.
‡ Not significant when compared to control values.
§ $P < 0.01$ when compared to control values.
‖ $P < 0.1$ when compared to control values.

6. E. J. Van Scott, R. Auerbach and G. D. Weinstein, *Archs Derm.* **89,** 550 (1964).
7. J. Almeyda, D. Barnardo and H. Baker, *Br. J. Derm.* **85,** 302 (1971).
8. J. R. Bertino, *Cancer Res.* **23,** 1286 (1963).
9. H. H. Roenigk, W. F. Bergfeld, R. St. Jacques, F. J. Owens and W. A. Hank, *Archs Derm.* **103,** 250 (1971).
10. D. G. Johns, A. T. Iannotti, A. C. Sartorelli and J. R. Bertino, *Biochem. Pharmac.* **15,** 555 (1966).
11. E. M. Hirsch, V. G. Wong, E. S. Henderson and E. J. Freireich, *Cancer, N.Y.* **19,** 600 (1966).
12. N. I. Berlin, *Ann. intern. Med.* **59,** 931 (1963).
13. R. V. P. Hatter, F. H. Shipkey and C. T. C. Tan, *Cancer, N.Y.* **13,** 288 (1960).
14. M. G. C. Dahl, M. M. M. Gregory and P. J. Scheuer, *Br. med. J.* **1,** 625 (1971).
15. E. H. Epstein and J. D. Croft, *Archs Derm.* **100,** 531 (1969).
16. H. V. Dubin and E. R. Harrell, *Archs Derm.* **102,** 498 (1970).
17. R. E. Olson, *Fedn Proc.* **30,** 131 (1971).
18. O. A. Iseri, L. S. Gottlieb, D. M. Hegsted and J. Vitale, *J. Lab. Invest.* **27,** 226 (1972).
19. G. G. de Pury and F. D. Collins, *Lipids* **7,** 225 (1972).
20. J. Folch, M. Lees and G. H. Sloane Stanley, *J. biol. Chem.* **226,** 497 (1957).
21. R. L. Searcy and L. M. Berquist, *Clinica chim. Acta* **5,** 192 (1962).
22. F. G. Soloni, *Clin. Chem.* **17,** 529 (1971).
23. G. R. Bartlett, *J. biol. Chem.* **234,** 466 (1959).
24. B. G. Stanley, G. E. Neal and D. C. Williams, *Biochem. Pharmac.* **18,** 159 (1969).
25. J. R. Guest and D. D. Woods, *Biochem. J.* **97,** 500 (1965).
26. C. C. Lucas and J. H. Ridout, *Prog. Chem. Fats* **10,** 1 (1967).
27. J. M. Buchanan, H. L. Elford, R. E. Loughlin, B. M. McDonald and S. Rosenthal, *Ann. N.Y. Acad. Sci.* **112,** 756 (1964).
28. M. A. Foster, K. M. Jones and D. D. Woods, *Biochem. J.* **80,** 519 (1961).
29. G. Bazzano, *Archs. intern. Med.* **124,** 710 (1969).