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EFFECT OF PRETREATMENT WITH METHOTREXATE ON THE REDUCTION OF DIHYDROHOMOFOLIC ACID IN MICE

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Abstract—Reduction of dihydrohomofolate and dihydrofolate was studied in normal and leukemic mice. Liver, kidney and spleen from mice treated with the compounds were analyzed by column chromatography on DEAE cellulose columns and the isolated compounds were characterized by ultraviolet absorption spectrum and chromatographic behavior. Both dihydrohomofolic and dihydrofolic acid were found to be reduced (*in vivo*) to the corresponding tetrahydro derivatives which were isolated from liver, kidney and spleen. The reduction was found to be blocked by pretreatment with methotrexate and the blocking effect was found to be time and dose dependent. The results of these studies strongly suggest that dihydrofolate reductase is responsible for the conversion of the dihydro derivatives to the corresponding tetrahydro forms. While it is likely that the conversion occurs in all tissues containing the enzyme, the possibility exists that distributional factors could account for the presence of tetrahydro derivatives in some tissues.

DIHYDROHOMOFOLIC acid (H_2HF), an analog of dihydrofolic acid (H_2F) containing one additional methylene group between the pteridine and *p*-amino-benzoic acid moieties, has been shown to be as effective a substrate of dihydrofolate reductase as H_2F and the product of the reaction, tetrahydrohomofolate (H_4HF), was found to be a potent inhibitor of partially purified *Escher ichia coli* thymidylate synthetase.[1] It was suggested that H_2HF might be effective against tumor in which resistance to methotrexate (MTX) was associated with high levels of H_2F-reductase.[1] In a preliminary study the antitumor activity of H_2HF was not definitive;[2] however, H_4HF was found to be a more effective antileukemic agent against a MTX-resistant tumor (L1210/FR-8) than the MTX-sensitive parent L1210.[2] The observations of Nahas and Friedkin[3] that H_2HF was not reduced to H_4HF in cells appeared to correlate well with the lack of apparent antitumour activity of H_2HF. Since H_4HF was found to be a significantly active antitumor compound, we have studied[4] its chemical changes *in vitro* and *in vivo* and found that mice injected with either H_4HF, or H_4HF oxidized to form H_2HF-like material by treatment with H_2O_2 or O_2, contained H_4HF in liver which was equal to > 99 per cent of the Bratton–Marshall-positive material[5] estimated in the liver. This observation suggested the possibility that H_2HF made synthetically from the reduction of homofolic acid might be reduced to H_4HF in the liver. The following report describes work which strongly suggests that the reduction of H_2HF to H_4HF occurs in mouse liver, spleen and kidney. A portion of this work has been reported elsewhere.[6]

MATERIALS AND METHODS

Homofolic acid (NSC-79,249), folic acid and methotrexate were obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute. Dithiothreitol (Cleland's reagent) was purchased from CalBiochem, and standard DEAE cellulose (0·88 m-equiv. binding sites/g) from Schleicher and Schuell, Inc. The dihydro derivatives of folate and homofolate were prepared by reduction with sodium dithionite.[7] The reduction of homofolate was carried out at temperatures between 18–20° and that of folate at 25–27°. The reaction mixture was kept covered with a layer of ligroin (b.p. 100–115°) to protect it from atmospheric oxidation. Male BDF_1 mice, 10–12 weeks old, weighing 20–25 g, maintained on Purina Chow and water *ad lib.*, were used in all the experiments. In some experiments mice bearing a 12-day-old MTX-resistant leukemia (L1210/FR-8) containing high levels of H_2F-reductase[8] were used.

H_2HF or H_2F (400 mg/kg) was given intraperitoneally (i.p.) in 2% sodium bicarbonate containing 0·6% sodium ascorbate and the mice were sacrificed at various time intervals. Methotrexate was administered in 2% sodium bicarbonate. The tissues (liver, spleen and kidney) and urine were analyzed by chromatography at 5° using a DEAE cellulose column (100 × 12 mm) prewashed with 200 ml of cold distilled water and 100 ml of the buffer to be used for elution. Tissues were homogenized (25% w/v) in the ice-cold buffer containing 0·01% dithiothreitol and centrifuged for 15 min at 5000 rpm in a refrigerated centrifuge. Three volumes of cold ethyl alcohol were added to the clear supernatant and the mixture was centrifuged again. Supernatant, containing 500–1000 μg equivalents of the drug as measured by Bratton–Marshall reaction,[5] was added to the column and eluted with 0·2 M ammonium acetate containing 0·01% Cleland's reagent for separating H_2HF and H_4HF. To separate H_2F and H_4F, the columns were eluted first with a linear gradient of tris buffer pH 7·6 (0·1 → 0·5 M, 100 ml of each concentration) containing 0·01% Cleland's reagent until 40 fractions (5 ml) were collected and then the molarity of the tris buffer was increased to 0·7 M until u.v.-absorbing material in the eluate became undetectable. The eluate from the chromatogram was monitored at 280 nm by an automatic recorder. The fractions were characterized by ultraviolet (u.v.) absorption spectra and amounts were estimated at the wavelength of maximum absorbance for each compound.

RESULTS

Reduced derivatives of folate and homofolate were always checked for purity by column chromatography and used only when they were > 95 per cent pure. H_2HF (400 mg/kg, i.p.) was given to BDF_1 mice and after 2 hr the mice were sacrificed and the livers were removed. The chromatogram of the liver extract is shown in Fig. 1. All the fractions from 30 to 50 showed the characteristic u.v.-absorption spectrum of H_4HF. No u.v.-absorbing material was detectable in fractions 50–80 where H_2HF was expected. Livers from untreated mice did not show any detectable amounts of u.v.-absorbing material in any fraction starting from fraction 30. This indicated that H_2HF was reduced to H_4HF in liver, probably by H_2F-reductase. The influence of MTX on the reduction of H_2HF in liver was examined next. The above experiment was repeated, except in this case the mice were pretreated with MTX (1·5 mg/kg, i.p.) 1 hr before H_2HF administration. The chromatogram of the liver extract is shown in Fig. 2. The fractions from 30 to 50 did not show the u.v. spectrum of H_4HF suggesting

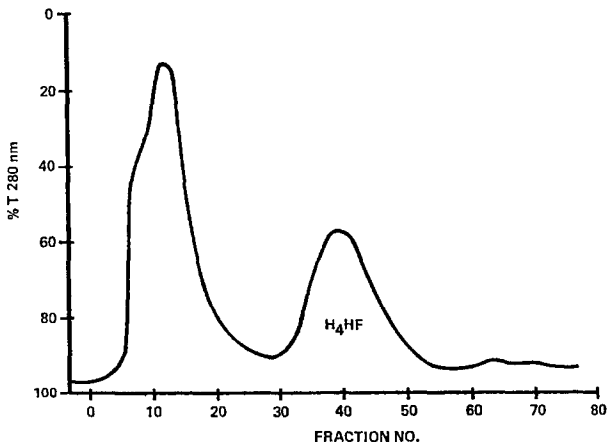

FIG. 1. Recovery of tetrahydrohomofolic acid (H_4HF) from mouse liver 2 hr after an intraperitoneal (i.p.) injection of dihydrohomofolic acid (400 mg/kg).

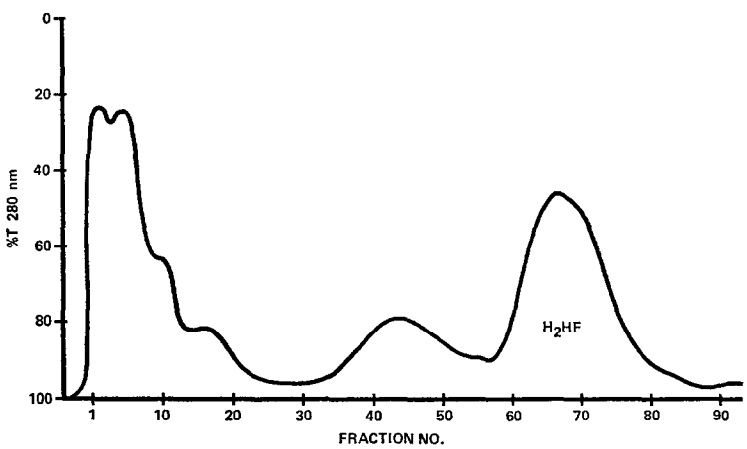

FIG. 2. Recovery of H_4HF and dihydrohomofolic acid (H_2HF) from mouse liver. Mice were given methotrexate (1·5 mg/kg) 1 hr before H_2HF (400 mg/kg) and sacrificed 2 hr later.

that no reduction of H_2HF had occurred. The fractions from 60 to 80 showed the spectrum of H_2HF indicating the presence of large amounts of unchanged H_2HF in the liver. These results revealed that the administered H_2HF recovered in the liver was not reduced due to blockade of the reduction mechanism by MTX. These experiments were repeated and mice were sacrificed at 1, 2 and 4 hr after H_2HF administration. The data summarized in Table 1 clearly indicate the reduction of H_2HF to H_4HF and its complete inhibition by MTX. Parallel experiments were conducted to determine if the sustained presence of H_4HF in liver for 4 hr after its administration[4] was dependent upon the reduction mechanism sensitive to MTX. The data summarized in Table 2 reveal that liver from MTX-pretreated mice showed large amounts of H_2HF, whereas H_2HF was not detectable in liver of mice without pretreatment. These results suggest

TABLE 1. REDUCTION OF DIHYDROHOMOFOLIC ACID IN MOUSE LIVER

Time (hr)	Reduced homofolates (μg/g liver)	Relative proportions H_2HF (%)	Relative proportions H_4HF (%)
A*			
1	310	Undetectable	98
2	220	Undetectable	98
4	108	Undetectable	95
B†			
1	248	93	7
2	113	100	
4	80‡		

* BDF_1 mice were injected with 400 mg dihydrohomofolic acid/kg and sacrificed at the time intervals indicated.

† Mice were pretreated with 1·5 mg methotrexate/kg 1 hr before the injection of dihydrohomofolic acid and sacrificed at time intervals indicated.

‡ Unidentified.

TABLE 2. FATE OF TETRAHYDROHOMOFOLIC ACID IN MOUSE LIVER

Time (hr)	Reduced homofolates (μg/g liver)	Relative proportions H_2HF (%)	Relative proportions H_4HF (%)
A*			
1	306	Undetectable	98
2	250	Undetectable	98
4	201	Undetectable	97
B†			
1	322	32	68
2	226	51	49
4	108‡		

* BDF_1 mice were injected with 400 mg/kg tetrahydrohomofolic acid and sacrificed at time intervals indicated.

† Mice were pretreated with 1·5 mg/kg methotrexate 1 hr before the injection of tetrahydrohomofolic acid and sacrificed at time intervals indicated.

‡ Unidentified.

that H_4HF oxidizes to H_2HF in cells and that a reducing system sensitive to MTX is necessary to regenerate H_4HF. In MTX-pretreated groups, when mice were sacrificed 4 hr after administration of either H_2HF (Table 1) or H_4HF (Table 2), neither H_2HF nor H_4HF was found in the liver. Instead some unidentified u.v.-absorbing material appeared in the fractions in which H_4HF was expected. This material showed a λ_{max} of 288–292 nm and was not characterized further.

TABLE 3. EFFECT OF METHOTREXATE (MTX) PRETREATMENT ON REDUCTION OF DIHYDROFOLIC ACID IN MICE

Experiments	Reduced folates* (μg/g liver)	Relative proportion H_2F (%)	Relative proportion H_4F (%)
1. 400 mg/kg H_2F i.p., sacrificed after:			
1 hr	190	0	100
2 hr	219	0	100
4 hr	111	0	100
2. Pretreatment with 1·5 mg/kg MTX, i.p.†			
1 hr	152	100	0
2 hr	148	9	91
3. Pretreatment with 15 mg/kg MTX, i.p.†			
2 hr	104	42	58
6 hr	103	36	64
12 hr	108	10	90
24 hr	122	10	90
4. Five daily injections of MTX, i.p. were given. H_2F(400 mg/kg) was given 24 hr after the last injection of MTX and mice were sacrificed after 2 hr.			
1·5 mg/kg × 5	113	0	100
15·0 mg/kg × 5	62	0	100
5. Twenty-four hr after pretreatment with MTX, i.p. at.†			
400 mg/kg	117	23	76
200 mg/kg	106	24	76
90 mg/kg	108	25	75
60 mg/kg	94	25	75

* Reduced folates/g of liver represents the sum of individual amounts of dihydrofolic acid (H_2F) and tetrahydrofolic acid (H_4F) as determined from the absorbance of the pooled fractions of the respective peaks of the chromatogram.

† After specified period of pretreatment with methotrexate, mice were injected i.p. with 400 mg/kg dihydrofolic acid and sacrificed after 2 hr.

Similarly, after injection of 400 mg/kg H_2HF the reduction of this compound in mouse liver was studied. The data are summarized in Experiments 1 and 2 of Table 3. The reduction of H_2F and its inhibition by pretreatment with MTX were similar to that observed with H_2HF, except that in mice pretreated with MTX (1·5 mg/kg, i.p.) 1 hr before H_2F administration and sacrificed after 4 hr, both H_4F (70 per cent) and H_2F (30 per cent) were found (not shown in Table 3). Next we attempted to determine the recovery period of the reduction mechanism after blockade by MTX-pretreatment at dose levels of 1·5 and 15 mg/kg as a single dose and as a daily dose for 5 days. Mice were given H_2F (400 mg/kg, i.p.) at various time periods after MTX treatment and sacrificed after 2 hr. The data are summarized in Experiments 2, 3 and 4. Complete recovery of the reduction mechanism was seen 2 hr after MTX treatment (1·5 mg/kg) using the amounts of H_4F relative to H_2F present in the liver as a criterion of recovery. However, after a higher dose of MTX (15·0 mg/kg) only 58 per cent recovery was seen. After pretreatment-periods of 6 and 12 hr the recoveries were 64 and > 90 per cent respectively. Since daily doses of MTX are several-fold more toxic than the

corresponding single dose, it was felt that after 5 daily injections inhibition of the reductase mechanism might persist up to 24 hr. Thus, two groups of 10 mice were injected with MTX, 1·5 and 15 mg/kg, i.p. daily for 5 days. On day six, 24 hr after the last injection, H_2F was injected and the mice were sacrificed after 2 hr. It can be seen that although there was less deposition of reduced folates in the livers of mice receiving the higher dose of MTX than those receiving the lower dose, the recovery of the reduction mechanism was 100 per cent. No H_2F and only H_4F was detectable on the chromatograms. It should be noted that although six out of ten mice died in the group treated with the higher dose of MTX and the other four mice were moribund at the time of injection of H_2F, the liver reduction mechanism for H_2F was not inhibited.

TABLE 4. REDUCTION OF DIHYDROHOMOFOLIC ACID IN LEUKEMIC MOUSE KIDNEY AND SPLEEN

Tissue source	Reduced homofolates (μg/g)	Relative proportions	
		H_2HF (%)	H_4HF (%)
Kidney*	925	38	62
Urine†	300	42	58
Spleen‡	160	40	60

* BDF_1 mice bearing a 12-day-old leukemia L1210/FR-8 were given 400 mg H_2HF/kg and sacrificed after 2 hr.
† In total urine.
‡ The leukemic mice were given 800 mg H_2HF/kg and sacrificed after 1 hr.

An attempt was also made to see if the inhibition of the H_2F reduction mechanism can be extended up to 24 hr by pretreatment with high doses of MTX. It can be seen (Experiment 5) that after a dose of 60 mg/kg there was 75 per cent recovery of the reduction mechanism, and this was essentially unchanged by further increases in doses up to 400 mg/kg.

Finally, we attempted to study the reduction of H_2HF in other tissues. In a preliminary study the reduction of H_2HF in mice bearing a MTX-resistant tumor (L1210/FR-8) was studied. It can be seen from the data (Table 4) that marked reduction of dihydrohomofolic acid did take place in kidney and spleen. Urine analysis also revealed more H_4HF than H_2HF excreted after 2 hr. These experiments suggest that reduction of H_2HF does occur in other organs.

Since H_2HF appeared to be a likely candidate for further evaluation as an antitumor agent, we also studied its stability under various conditions so that it could be suitably formulated. It was found that H_2HF was stable after incubation at 37° for 1 hr in the absence of any reducing agent (Table 5). Autoclaving H_2HF in 0·6% ascorbate solution for 15 min at 15 lb pressure decomposed more than 50 per cent of

TABLE 5. STABILITY OF DIHYDROHOMOFOLIC ACID (H_2HF) UNDER VARIOUS CONDITIONS

Experiments	Recovery of H_2HF after DEAE cellulose column chromatography (%)
Five mg/ml H_2HF in 0·1 M ammonium bicarbonate was incubated at 37° for 1 hr	98
Five mg/ml solution in 0·6% ascorbate	98
Autoclaved at 15 lb pressure for 15 min	49
Forty mg/ml H_2HF in 0·6% ascorbate kept at 25° for 2 hr	85
Kept at 25° for 17 hr	22
Kept at 4° for 17 hr	52
Kept at −30° for 17 hr	83
Kept at −80° for17 hr	97
Kept at −80° for 16 weeks	96

the drug. A solution (40 mg/ml) in 0·6% ascorbate was found to decompose at room temperature (25°) as well as at 4° within 17 hr. The solution, kept below −30°, was stable for 17 hr, and at −80° was stable for more than 6 months.

DISCUSSION

Data presented in this report have clearly demonstrated that after i.p. administration H_2HF was reduced to H_4HF which was isolated from mouse organs, namely liver, spleen and kidney, and the reduction mechanism was found to be sensitive to MTX-pretreatment. In parallel studies H_2F was found to be reduced *in vivo* and the reduction was also sensitive to MTX-pretreatment. The amounts of both the compounds taken up by the liver and reduced were comparable. Nahas and Friedkin[3] did not find reduction *in vitro* of H_2HF by leukemic cells. The difference between their results and the present findings may be partly due to differences in experimental conditions and partly due to the possibility of subtle differences between the biochemical properties of isolated leukemic cells and the leukemic spleens, kidney and normal liver used in the present study. We have previously reported[4] that H_4HF is present in the liver for 4 hr after i.p. administration in mice, and that no H_2HF could be detected during this period. It was felt that H_2F-reductase activity in the liver might have been responsible for the presence of H_4HF and absence of H_2HF. Data presented here clearly show that when reductase activity was blocked by MTX-pretreatment a large amount of H_2HF was recovered in the liver after H_4HF administration, indicating the presence of a continuous oxidation–reduction system for H_4HF in cells.

The question now arises as to which diastereoisomer of H_4HF is responsible for tumor inhibition. Kisliuk and Gaumont[9] have shown that *d*,L-isomer of H_4HF with the "unnatural" configuration at carbon 6 is responsible for the inhibition of *S. faecium* growth and that the *l*,L-isomer is inactive. Since H_4HF formed, as a result of enzymic reduction, is the *l*,L-isomer[9] we anticipated that H_4HF formed *in vivo* after administration of H_2HF must be the *l*,L-isomer. Also since continuous oxidation and reduction of H_4HF does take place in cells, even if chemically prepared *dl*,L H_4HF is administered, the eventual formation of the *l*,L diastereoisomer from the *d*,L form in

cells is a likely possibility. If the *d*,L diastereoisomer is considered to be responsible for antitumor activity, the cells with greater H_2F-reductase activity might regenerate more *l*,L form and, therefore, be less sensitive to H_4HF treatment. However, Mead *et al.*[2] have shown H_4HF to be more effective in leukemia containing high levels of H_2F-reductase than its parent line. Recently our preliminary study[6] has shown that H_2HF also has antitumor activity comparable to H_4HF against leukemia L1210/FR-8 containing high levels of H_2F-reductase. These observations suggest that *l*,L diastereoisomer of H_4HF could be responsible for the antitumor activity in a mammalian system. In this respect it has been shown that enzymically generated *l*,L tetrahydrohomofolate is twice as inhibitory as chemically prepared *dl*,L-tetrahydrohomofolate against *E. coli* thymidylate synthetase.[10]

It is interesting to note that pretreatment with MTX was followed by the breakdown of reduced homofolates in liver 4 hr after injection. It appears that the stability of H_4HF and H_2HF is not only dependent on H_2F-reductase but possibly on protective mechanisms which prevent chemical breakdown *in vivo*. The stability of reduced homofolates in mice pretreated with MTX needs to be studied further. Studies on the reduction mechanism using H_2F, a natural substrate for H_2F-reductase, have indicated that within 2 hr after a dose of MTX (1·5 mg/kg), the reduction of H_2F to H_4F in liver had returned to control levels and after a higher dose (15 mg/kg) the recovery of H_2F-reductase activity was found to be 58 per cent. Neither daily doses (15 mg/kg $\times$ 5) for 5 days nor a single dose up to 400 mg per kg extended the inhibition of H_2F-reductase up to 24 hr. Condit and Mead[11] have also reported that in mice pretreated with MTX 24 hr before H_2F administration, the increase in citrovorum factor activity in liver was unaltered. It has been reported that H_2F can displace about 13% of methotrexate retained in man for extended periods.[12] It seems likely, therefore, that the recovery of the enzymic activity observed in these studies could be related to the displacement of methotrexate from the enzyme by the large doses of H_2F and H_2HF which were employed. Alternatively, a distribution of the tetrahydro derivatives of the folates to the various tissues could explain the apparent recovery of the enzyme activity from inhibition by methotrexate.

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