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TOLERANCE OF LONG-TERM METHOTREXATE INFUSIONS BY MICE

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Abstract—Mice were chronically exposed to between 1×10^{-8} and 5×10^{-9} M plasma methotrexate (MTX) for 2 months. No signs of toxicity were manifested except minor facial hair loss. At the end of this time they were sacrificed, and the activity of dihydrofolate reductase in crude Tris extracts of small intestine, femur, liver and kidney was determined at pH 7.0 and 8.6. At pH 7.0 the enzyme activity was maintained at control levels in small intestine and femur but was decreased to half of controls in liver and kidney. The enzyme activity at pH 8.6 was increased significantly above controls in small intestine and femur but remained at control levels in liver and kidney. These results suggest that some organs adapt to the chronic presence of MTX by increasing their enzyme synthesis rate while other organs are able to function normally for prolonged periods of time with less than normal amounts of active enzyme.

Recent evidence has indicated that long-term exposure of mice to very low concentrations of methotrexate (MTX) can be toxic. Under these conditions the time of exposure is a critical factor [1, 2]. Concepts derived from these and earlier studies by Hakala [3], Werkheiser [4, 5] and others [6–9] suggest that the primary parameters determining tissue sensitivity to methotrexate are rate of drug entry, degree of drug binding to the enzyme dihydrofolate reductase (DHFR) [5, 6, 7, 8-tetrahydrofolate: $NAD(P)^+$ oxidoreductase EC 1.5.1.3], and rate of new DHFR synthesis.

We have done experiments which indicate that mice can be exposed to between 1×10^{-8} and 5×10^{-9} M plasma methotrexate by constant infusion for months without any apparent toxicity, as judged by weight loss and external appearances. However at plasma methotrexate concentrations of 2×10^{-8} M or higher, maintained for 3–4 days, many mice die [1]. This type of data suggests that there is a very critical balance between drug entry, drug binding with inactivation of some enzyme, and new enzyme synthesis rate. We have conducted these experiments to determine whether a more rapid than normal enzyme synthesis rate could be the mechanism by which mouse tissues tolerate a low constant methotrexate exposure.

THEORETICAL CONSIDERATIONS

Normally in the steady state *in vivo* there is a balance between synthesis and attrition such that DHFR activity in various tissues remains constant within normal limits imposed by diet, diurnal and seasonal variation. If, however, methotrexate is constantly present in the extracellular fluid, a new steady state is established. In such a new steady state, the conditions illustrated in Fig. 1 most likely exist. In response to an initial decrease in active enzyme due to methotrexate binding, there may be an increase in enzyme synthesis rate so that active enzyme remains constant. On the other hand there may be no change or even an inhibition of enzyme synthesis rate because of the potential effect of methotrexate on protein synthesis through its effects on purine and RNA synthesis. Considering these alternatives we might expect the following, after exposing mice to methotrexate at a constant level for 2 months. If an increase in rate of enzyme synthesis occurs to compensate for enzyme removed by methotrexate binding, then the amount of active enzyme measured in tissues from these mice should be similar to that from tissues of mice not receiving methotrexate. If no change or a decrease in enzyme synthesis occurs, then the amount of active enzyme in tissues of treated mice would be less than that in tissues of normal mice. In both situations if methotrexate is indeed entering cells and binding to enzyme, the total amount of enzyme present (free plus bound) should in the experimental mouse tissues be equal to or greater than that in the control mice. We make the assumption that enzyme activity measurements *in vitro* in the presence of methotrexate at a pH of 7.0 give us an estimate of relative free enzyme activity *in vivo*. It has been reported that the dissociation of the methotrexate–DHFR complex is greater at a pH of 8.6 than 7.0 [10]. Therefore, remeasurement of enzyme activity with comparison to controls at a pH of 8.6 gives us an indication as to whether methotrexate was indeed entering the cell and binding to enzyme. This enzyme activity at a pH of 8.6 would then represent free enzyme activity plus the enzyme activity released from methotrexate binding by the shift in pH. We refer to this activity as total enzyme activity. It is probably an underestimate of the total enzyme *in vivo*, since recovery *in vitro* of bound enzyme activity is less than 100 per cent and varies with the quantity of methotrexate present (see Fig. 3 and accompanying text). However, absolute recoveries have little bearing on interpretation of results which are based on relative comparisons.

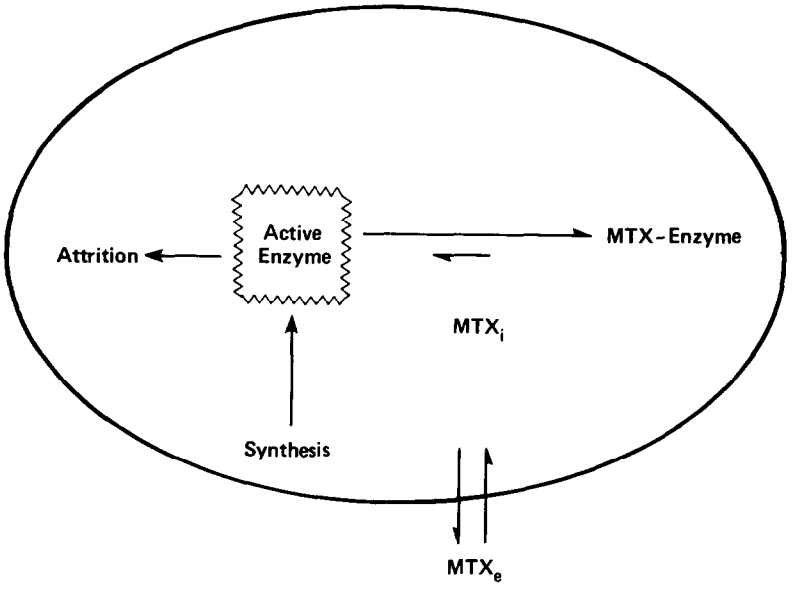

Fig. 1. Schematic diagram to illustrate the steady state condition after chronic exposure to MTX. Subscript i = intracellular, e = extracellular.

EXPERIMENTAL

General approach

Six normal CDF_1 male mice were implanted subcutaneously with an MTX-containing infusion cell which put out drug *in vitro* at 37° at approximately 0.2 μg/hr [11]. If replaced every 2 weeks, this infusion cell maintained the plasma MTX constant between 1×10^{-8} and 5×10^{-9} M (see Fig. 2). Six control mice were treated simultaneously with infusion cells containing no drug. The mice were weighed daily and were decapitated after 2 months. Blood was collected in heparinized tubes; plasma was separated and frozen for future MTX assay. Tissues were removed and blotted, and aliquots were dropped into liquid nitrogen. These samples were then weighed, wrapped and stored at −15° for future enzyme assay.

Other aliquots of tissue including the lung, heart, small intestine, kidney, liver, spleen, pancreas, skin, lymph nodes and thymus were fixed in 10% buffered formalin, sectioned at 6 μm and stained with hematoxylin and eosin for pathological analysis.

Plasma MTX assay

Plasma was assayed for its methotrexate concentration by an enzymatic assay [12], which is based on the principle that dihydrofolate reductase converts dihydrofolate to tetrahydrofolate in the presence of NADPH and is prevented from so doing to a degree determined by the quantity of methotrexate present. Dihydrofolate reductase from L1210 leukemia cells was used as the enzyme source in this assay. In the manner used, the assay was sensitive to methotrexate plasma concentrations as low as 1×10^{-9} M.

Tissue preparation

The frozen tissues were homogenized in 2 ml of 0.1 M Tris buffer (pH 7.4) within 2–3 days. Aliquots of tissues used ranged from a low of 0.07 g for femurs to a high of 0.45 g for liver. They were centrifuged at 56,000 *g* at 4° for 30 min, and both the clear supernatant and the precipitant were refrozen. The supernatants were thawed and divided into aliquots for DNA, protein and enzyme activity determinations. Protein was measured in the supernatants by the technique of Lowry *et al.* [13] using bovine serum albumin as the standard. DNA was determined in both the supernatant and precipitant fractions by a modified Schneider method [14, 15]; calf thymus DNA type 1 (Sigma) was used as the standard.

Tissue DHFR assay

DHFR activity measurements were performed on the Tris supernatants at 37° with dihydrofolate as substrate at two pH values: 7.0 and 8.6. A spectrophotometric method based on the decrease in absorbance at 340 nm was used [16]. Initial rates over the first 5–10 min were determined by absorbance readings made at 6-sec intervals on a Gilford automatic recording spectrophotometer. Specific activities are expressed as nmoles of dihydrofolate reduced/h/mg of protein; a combined extinction of 12,300 M^{-1} cm^{-1} was used [17]. The standard assay mixture in a total volume of 1 ml contained 0.12 m-mole Tris buffer (either pH 7.0 or 8.6), 0.17 m-mole KCl, 30 nmoles dihydrofolate, 130 nmoles NADPH and approximately 4 μmoles of 2-mercaptoethanol. The reaction was initiated by adding 0.025 to 0.10 ml of tissue extract. Reaction blanks with the standard assay mixture minus dihydrofolate were always run concomitantly with each sample tested for DHFR activity. The reported DHFR value is the total activity minus the NADPH oxidase activity in the absence of dihydrofolate.

The small intestine Tris supernatant initially had so much NADPH oxidase activity that the DHFR activity was not possible to quantitate. However, with several steps of refreezing, thawing and centrifuging, sufficient oxidase activity was removed so that quantitation of DHFR activity in this crude extract was

possible. Although repeated freezing and thawing also cause a decrease in DHFR activity, controls and experimental samples were treated identically so that relative comparisons should be valid. The intestinal NADPH oxidase activity at the time of DHFR quantitation represented approximately 50 per cent of the total optical density change. The other tissue Tris extracts (femur, kidney and liver) have less NADPH oxidase activity than the small intestine extract and, therefore, repeated freezing and thawing were not necessary to quantitate DHFR activity in these samples.

MTX titration of control tissues

Each control tissue was titrated with MTX at both pH 7.0 and 8.6 in addition to measuring DHFR activity at these two pH values. This was done in order to determine the quantity of MTX-bound enzyme activity at pH 7.0 that could be recovered at pH 8.6.

RESULTS AND DISCUSSION

Gross and histopathological animal changes

The animals' body weight change was no different between controls and experimentals. In all respects except one, the experimental mice appeared normal. The one exception was the loss of facial hair. This could have been due to the reduced activity of DHFR in the liver with a consequent reduction in the supply of reduced folate coenzymes to the hair follicles. This would suggest that the hair follicles depend on an external supply of reduced folate coenzymes for normal function.

Histopathological evaluation of hematoxylin-and eosin-stained sections resulted in no abnormal definitive drug-induced alteration of the organs or tissues examined. The skin and subcutaneous tissue adjacent to the cell dispensing site were free of tissue reaction.

Protein and DNA

Results indicated no detectable differences ($P > 0.05$) between experimental and control tissue content of DNA in homogenates or protein in the Tris extracts. This is not surprising, since the animals appeared normal. The DHFR probably represents such a small per cent of the total protein that changes in DHFR content are not detectable by total protein determination.

Plasma methotrexate

Plasma methotrexate concentrations are illustrated in Fig. 2. The mean methotrexate plasma concentration of the six experimental mice at the time of sacrifice (60 days) was $5.25 \pm 1.17 \times 10^{-9}$ M (mean ± standard error). Additional mice were sacrificed at various intervals during the 2 months of exposure to determine whether plasma methotrexate was constantly maintained. Analysis of variance of the means of groups of six mice indicated that when mice were sacrificed 2 weeks after cell implant the differences among means was statistically insignificant ($P > 0.05$). However, the mean values obtained 2–4 days after cell implant were significantly different from those obtained 2 weeks after cell implant. Further investigation indicated that this difference was due to a slight decrease in infusion cell output with residence time *in vivo*. These data indicate that plasma concentration was maintained in the mice for 2 months between 1×10^{-8} and 5×10^{-9} M methotrexate.

Active enzyme at pH 7.0

The data in Table 1, columns 1 and 2, indicate that the free enzyme activity in the four organs sampled was similar in experimentals and controls except for kidney and liver. Kidney was 48 per cent of control and liver was 41 per cent of control. The conclusion based on our prevous theoretical considerations is that kidney and liver do not increase their rate

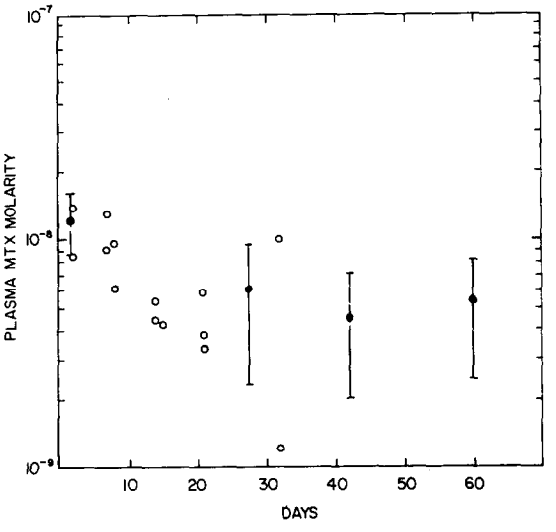

Fig. 2. Plasma methotrexate concentrations in mice at various times after subcutaneous implantation of infusion cells. The cells were replaced every 2 weeks. The clear circles represent plasma values for individual mice. The filled circles represent the mean ± S.D. for six mice.

Table 1. Effect of chronic 2-month MTX exposure on DHFR activity in several mouse tissues*

Type of tissue	DHFR activity (nmoles FH_2 converted/hr/mg protein) Free enzyme (pH 7.0) Control	Experimental	Total enzyme (pH 8.6) Control	Experimental
Small intestine	11.71 ± 1.95	11.71 ± 4.4	12.20 ± 1.95	38.54 ± 7.8†
Femur	87.32 ± 7.32	79.02 ± 6.34	96.58 ± 5.85	149.76 ± 8.29†
Kidney	353.17 ± 53.66	167.80 ± 26.83†	447.32 ± 42.93‡	477.56 ± 56.58
Liver	132.68 ± 12.68	54.63 ± 4.88†	180.00 ± 15.12‡	176.58 ± 6.34

* Mean ± S.E.M., N = 5 or 6. † $P \leq 0.05$, experimental different from control. ‡ $P \leq 0.05$, control at pH 7.0 different from control at pH 8.6.

of enzyme synthesis during this new steady state with constant MTX exposure.

Total enzyme at pH 8.6

Controls. Figure 3 illustrates a typical MTX titration of DHFR activity in a control tissue extract *in vitro* at pH 7.0 and 8.6. The data indicate that between 75 and 85 per cent of the enzyme activity that is inhibited at pH 7.0 is measured at pH 8.6. It can be seen that this recovery varies with the concentration of MTX present in the cuvette. At low MTX ($< 1 \times 10^{-9}$ M) concentrations, the DHFR recovery is greater than at higher MTX concentrations. All control tissue extracts were titrated with MTX in a way similar to that illustrated in Fig. 3 at pH 7.0 and 8.6 with respect to their DHFR activity. All extracts had an equivalent amount of enzyme activity inhibited at least 50 per cent at pH 7.0 by 1×10^{-9} M methotrexate in the assay cuvette. The recovery of enzyme activity at pH 8.6 that was inhibited at pH 7.0 between 1×10^{-9} and 5×10^{-9} M was as follows: liver 50 per cent, kidney 50 per cent and femur 40–80 per cent.

Comparison of experimental and control. The data in Table 1, columns 3 and 4, indicate that the total enzyme activity in the small intestine and femur was higher in experimentals than controls. These data, considered along with the finding that free enzyme was similar in experimentals and controls, suggest that these organs can increase their rate of new enzyme synthesis to maintain constant levels of active enzyme in the presence of constant MTX. The liver and kidney total enzyme activity was similar in experimental and controls and these data verify that the decrease in free enzyme activity at pH 7.0 in experimentals as compared to controls was most likely due to the presence of MTX. These tissues, therefore, are unable to increase their new enzyme synthesis rate sufficiently to overcome this decrease in active enzyme. Studies by Ngu *et al.* [18] suggested that in rat liver the level of DHFR normally present represents the maximal level obtainable. Since we saw neither in the animals nor in the tissues any gross or pathologically discernable toxicity, either these organs are able to function normally with less than normal DHFR or toxicity is subtle and takes more than 2 months to manifest itself.

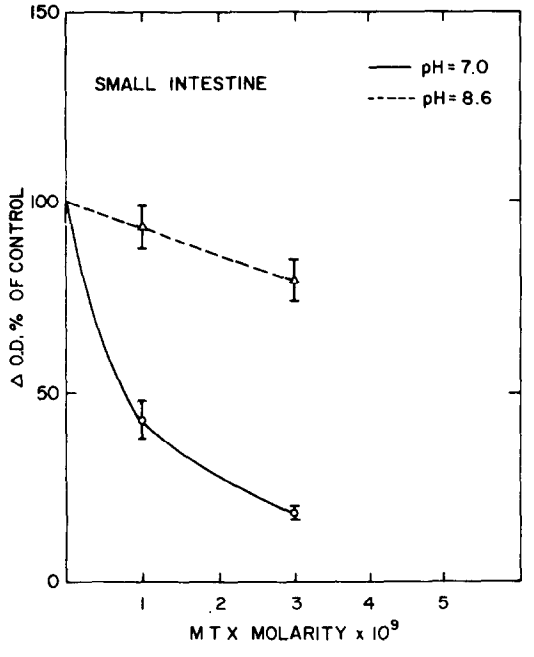

Fig. 3. DHFR activity and its inhibition by MTX in normal mouse small intestine. The activity of control Tris extracts was titrated *in vitro* against MTX using the DHFR assay described in the Experimental section. Mean ± S.E.M. with $N = 5$ or 6.

Comparison of enzyme activity at pH 7.0 and 8.6

The DHFR activity in the control tissue extracts at pH 7.0 was similar to the activity at pH 8.6 in small intestine and femur; it was significantly higher ($P \leq 0.05$), however, at pH 8.6 in the kidney and liver. This could be due to the presence of significant pools of reduced forms of folic acid in our extracts, which have been reported to inhibit DHFR activity to a greater extent at neutral pH than at higher pH [19]. This phenomenon, although interesting in itself, should not influence our interpretations, which are based on relative comparisons of experimental with control animals at the two pH values.

We conclude from the above comparisons that the maintenance of free enzyme activity at the same level in intestine and femur of experimental animals as in control animals is the result of increased enzyme synthesis. MTX must have entered the cell, since there was an increase in total enzyme activity in these two organs relative to controls. In contrast, kidney and liver did not exhibit an increase in total enzyme activity. This suggests no increase or a decrease in enzyme synthesis in these organs under the conditions of constant MTX exposure. There was some question as to whether the decrease in free DHFR in the liver and kidney could have been due to the binding of extracellular MTX to enzyme during the homogenization *in vitro* of experimental tissue because of the high levels of MTX in bile and urine. Calculations indicated that these factors would not contribute significantly. However, an additional experiment was conducted to check out this possibility. Twelve mice were treated identically as before except that they were sacrificed 24 hr after stopping the methotrexate infusion. By this time the plasma MTX was undetectable and pharmacokinetic calculations indicated that urine and bile methotrexate concentrations would also be below detectable levels. The measured enzyme activity at pH 7.0 of experimental liver and kidney extracts was 54 and 67 per cent of controls respectively. These results were not significantly different from results of the previous experiment in which methotrexate was infused until the time of sacrifice. The slightly higher values of measured free enzyme activity, although not statistically significant (compare to previous values of 41 and 48 per cent of controls for liver and kidney, respectively), suggest some titration of enzyme by methotrexate in bile and during homogenization procedures. However, as suggested by pharmacokinetic calculations it is an insignificant amount under these experimental conditions.

In summary, mice were able to tolerate chronic exposure of MTX without any adverse manifestations of toxicity except some hair loss. This adaptation

appeared to be due to several mechanisms. The femur and small intestine maintained a constant amount of free enzyme activity in the presence of chronic MTX whereas the liver and kidney were unable to do so. Increased enzyme synthesis rate is suggested as one mechanism of tolerance. Ability to function adequately with less than normal amounts of enzyme is also suggested as a mechanism of tolerance to chronic methotrexate exposure.

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