Pharmacokinetic Basis for Differences in Methotrexate Sensitivity of Normal Proliferative Tissues in the Mouse

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ABSTRACT

Following s.c. administration of varying doses of methotrexate (12 to 400 mg/kg) to mice, the drug accumulated more rapidly and to much higher levels in the small intestine in comparison to bone marrow. The persistence of exchangeable levels of drug (i.e., above that presumed to be equal to the dihydrofolate reductase-binding capacity) was also much greater in the small intestine. In addition, the more prolonged persistence of exchangeable drug in the small intestine compared to marrow correlated with a substantially longer duration of inhibition of DNA synthesis ([6-3H]deoxyuridine incorporation) in the former. Earlier recovery of DNA synthesis as a consequence of more rapid loss of drug appears to explain the lower sensitivity of marrow compared to small intestine to the effects of this agent in mice. These studies extend prior studies in our laboratory to the two major host proliferative populations in mice and allow us to propose that the property for accumulating and maintaining pharmacologically effective intracellular levels of folate analogs is differential among all proliferative tissues (tumor and normal) of this animal and probably in higher mammals as well.

INTRODUCTION

The cytotoxic action of 4-aminofolate analogs, which mediate their effects through inhibition of dihydrofolate reductase (21), is usually attributed (7, 9, 21) to the ultimate effect on the synthesis of DNA. The magnitude of the cytotoxicity observed at least in murine tissues is a result (2, 9, 11, 14–18) of the extent and, perhaps more importantly, the duration of the suppression of DNA synthesis. The eventual recovery of DNA synthesis is related to the rapidly reversible nature of the enzyme blockade in situ (6, 9, 11, 15, 22). The greater cytotoxic effects observed (5) on proliferative epithelial cells in small intestine, the drug-limiting organ in mice, compared to effects on proliferative elements in bone marrow appear to be associated (2) with an earlier recovery of DNA synthesis in the latter. In our own studies, selective antitumor effects of therapeutic doses of folate analogs in responsive murine tumor models have also been associated (14–18) with an earlier recovery of DNA synthesis in small intestine compared to tumor cells following pronounced inhibition in each tissue. The difference observed in the duration of inhibition of DNA synthesis between tumor cells and normal proliferative intestinal epithelium has been related (14–18) to a more prolonged persistence of freely exchangeable (osmotically active) drug in tumor cells at levels which are required (6, 12, 15, 20) to effectively inhibit dihydrofolate reductase.

While evidence for a pharmacokinetic basis has been derived (14–18) for the difference in the duration of inhibition of DNA synthesis in mouse small intestine compared to tumor cells, similar evidence has not been provided to explain the same difference between proliferative intestinal epithelial cells and marrow. In the present report, we now provide data on the time course for accumulation and persistence of methotrexate in these normal proliferative mouse tissues following administration in vivo. We also show other data on the time course for inhibition and recovery of DNA synthesis in each case. Our results reveal a dramatic difference in the pharmacokinetic behavior of methotrexate in these tissues. The accumulation and persistence of exchangeable methotrexate in marrow were markedly reduced compared to small intestine, and this difference correlates with a much shorter duration of inhibition of DNA synthesis in the former case. These results extend the conclusion derived from our earlier (2–19) studies to both normal proliferative cell populations in the mouse. They also provide a well-documented basis for understanding the greater sensitivity of small intestine compared to bone marrow in this animal to this folate analog which is not ordinarily seen for other mammals (5) and patients (1, 4) and has remained unexplained for 30 years.

MATERIALS AND METHODS

General. Mice used during these studies were C57BL/6 x DBA/2 F1 (hereafter called B6D2F1) females weighing 20 ± 0.5 (S.D.) g and obtained from ARS Sprague-Dawley, Madison, Wis. Methods of analysis for drug in tissue and plasma were already described (12, 13–19). After s.c. administration of drug, mice were sacrificed by cervical dislocation. The small intestine was surgically removed and placed in cold (0°) 0.14 M NaCl-O.02 M sodium phosphate, pH 7.4. The organ was sectioned, opened longitudinally, and cleaned. After blotting to remove excess liquid, the tissue was weighed and homogenized, and drug was removed by heat extraction for analysis. The average weight of small intestine in control mice was 0.94 ± 0.07 g, wet weight, per mouse. The total holding period in buffered 0.14 M NaCl-O.02 M sodium phosphate during washing is maintained for at least 5 min in order to allow for drug removal from interstitial space and blood vessels. Further details relating to the adequacy of this procedure for measuring intracellular drug have been discussed elsewhere (12, 18). Blood was obtained by bleeding from the orbital sinuses by means of a micropipet. Samples were allowed to clot, and serum was collected following centrifugation. Analysis for drug was carried out after heat treating and clarifying samples by centrifugation. Marrow was collected by aspiration from the femur after surgical removal from the animal. Cold (0°) 0.14 M NaCl-O.02 M sodium phosphate was forced through one end of the femur with the aid of a syringe, and the cell suspension...
was collected from the opposite end. Marrow from both femurs from 4 to 8 mice was pooled, and RBC were removed by resuspending the marrow in cold 0.17 M NH₄Cl and by washing and resuspending the remaining cells in cold 0.14 M NaCl-0.02 M sodium phosphate. The average amount of marrow recovered from control mice was 23.3 ± 3.9 mg, wet weight, per mouse. Dihydrofolate reductase content of the tissue was determined by direct titration with folate analog (methotrexate or methasquin) in a manner similar (13) to that described originally by Werkheiser (20). Estimates of acute lethal toxicity were obtained by s.c. administration of varying doses of methotrexate to mice which were held for a total of 3 weeks. Animals dying of toxicity showed a 30% weight loss and gross evidence of pathology in the small intestine. There were no detectable differences in total cellularity or cellular morphology of bone marrow when marrow from control and drug-treated animals was examined microscopically following Wright-Giemsa staining.

**Measurements of DNA Synthesis.** The rate of DNA synthesis in small intestine and marrow in control and drug-treated animals was determined by measurements of the extent of \[\text{\textsuperscript{3}H}\text{-dUrd}\] incorporation into DNA. Usually, 2 to 10 \(\mu\)Ci of \[\text{\textsuperscript{3}H}\text{-dUrd}\] (0.2 ml of 0.2 mM solution) were administered i.p., and animals were sacrificed after 5 min. The DNA was extracted (6), and the radioactivity was determined by liquid scintillation counting. The incorporation of radioactivity into DNA of small intestine and marrow cells was usually linear for at least 20 min after \[\text{\textsuperscript{3}H}\text{-dUrd}\] administration. A standard curve for control rates of incorporation for intervals of 10 min between 0 and 40 min was always derived for each experiment to verify linear rates. Values for relative rates of incorporation (drug-treated versus control mice) were derived from an average of individual determinations (dpm/mg DNA) in 3 to 5 mice. These are calculated as percentage of control values (dpm/10 min/mg DNA of 8 to 10 \(\times 10^6\) for small intestine and 5 to 7 \(\times 10^6\) for marrow after the injection of 2 \(\mu\)Ci \[\text{\textsuperscript{3}H}\text{-dUrd}\]). Values for specific incorporation into DNA in drug-treated animals are measurable to about 0.1% of control values. These values represent absolute incorporation rates which are approximately 30% above the level of radioactivity estimated to be present as a result of nonspecific binding to acid-soluble cellular constituents. This base-line value was derived in 2 different ways: (a) mice were pulse labeled with \[\text{\textsuperscript{3}H}\text{-dUrd}\] 2 hr after a flooding dose (2000 mg/kg) of antifolate, and tissues were processed in the manner already indicated; and (b) \[\text{\textsuperscript{3}H}\text{-dUrd}\] was added directly to a cold (0°C) homogenate of each tissue. The amount of \[\text{\textsuperscript{3}H}\text{-dUrd}\] added was equivalent to the total radioactivity found in tissue prior to trichloroacetic acid treatment after in vivo administration of \[\text{\textsuperscript{3}H}\text{-dUrd}\]. Processing of the tissue homogenate was then carried out in the usual manner. In either case, the level of radioactivity found is usually about 0.2 to 0.4% of the total incorporation during a 10-min interval in controls. All values for total incorporation are corrected for this baseline value. Fluctuation in intracellular pool size of deoxyuridine in drug-treated animals does not appear to be a major limitation for measurements of valid incorporation rates. The administration of the same amount of radioactivity at a \[\text{\textsuperscript{3}H}\text{-dUrd}\] dosage (0.2 ml of 2 mM solution) 10-fold higher than the dosage normally used gives essentially the same result when comparisons are made between control and drug-treated animals. This would suggest that the amount of \[\text{\textsuperscript{3}H}\text{-dUrd}\] accumulated intracellularly as \[\text{\textsuperscript{3}H}\text{-dUMP}\] is well in excess of the existing pool of nucleotide in either group.

**RESULTS**

Reevaluation of Methotrexate Tolerance and Plasma Pharmacokinetics in Mice. The B6D2F1 mice used during these studies exhibited a higher tolerance to folate analogs than was reported (19) for this same inbred strain during our previous studies. Acute lethality of methotrexate in mice used during the current study was only observed at doses above 100 mg/kg. The average value for a 50% lethal dose derived during the entire period of these more recent studies was 386 ± 65 mg/kg as compared to our previously reported (19) value of 125 ± 30 mg/kg. Although considerable amounts of data on methotrexate pharmacokinetics and inhibition of DNA synthesis were derived for mouse small intestine in our prior studies (14, 16-18), in view of this shift in tolerance, it was necessary to do a parallel study in small intestine and marrow. For the same reason, data on clearance for methotrexate were also obtained during these studies. Levels of methotrexate in plasma at various times after the s.c. administration of 12 and 400 mg/kg are shown in Chart 1. For purposes of comparison, data derived during our earlier studies (18) at these same doses are also shown in the same chart. The relative relationship observed between plasma level and dosage appears to be approximately the same for both sets of data. Initial plasma levels and the rate of decrease in drug level during the phase (12 to 24 hr after drug administration) believed to be due to reabsorption from small intestine (8) appear to be similar for both studies. However, the rate of clearance during the phase (1 to

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2 The abbreviations used are: \[\text{\textsuperscript{3}H}\text{-dUrd}\], \([\text{\textsuperscript{3}H}]\text{-deoxyuridine}\).
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10 hr after drug administration) believed to be predominantly excretory (8) appeared to be more rapid in mice which were used during the more recent study.

**Methotrexate Pharmacokinetics in Mouse Small Intestine and Marrow.** The rate of initial accumulation and the time course for accumulation and persistence of methotrexate in both small intestine and bone marrow were derived for single methotrexate doses between 12 and 400 mg/kg s.c. The rate of initial accumulation of drug in tissue is shown in Chart 2. Since accumulation of drug in both tissues (Charts 3 and 4) occurred at a constant rate for at least 10 min, it was possible to compare dose dependence for initial rates of accumulation in these tissues. Initial rates of accumulation in small intestine were much more rapid than in marrow. Also, while the initial rate for small intestine continued to increase at doses up to 400 mg/kg, very little increase in accumulation occurred in marrow at doses above 100 mg/kg. A similar relationship between initial accumulation in small intestine and tumor cells had already been reported (14, 17, 18) from our laboratory. Maximum levels of accumulation at every dose studied were also considerably greater in small intestine (Chart 3) than in marrow (Chart 4). However, maximum accumulation occurred in small intestine (~1 hr) much later than in marrow (~15 min).

The persistence of methotrexate in both tissues was also dramatically different. In small intestine, exchangeable levels (not bound to dihydrofolate reductase) of drug persisted for 5 to 6 hr after 12 mg/kg, 12 to 16 hr after 48 mg/kg, 16 to 24 hr after 96 mg/kg, and 36 to 48 hr after 400 mg/kg. In marrow, however, exchangeable levels of drug persisted for only 0.5 to 1 hr, 1 to 2 hr, 3 to 4 hr, and 5 to 6 hr after the same dosages. During these studies, because of the small amount of marrow available in mice, the value for the drug-binding equivalence of the initial dihydrofolate reductase level in tissue could only be confirmed for small intestine by direct titration with antifolate of enzyme in cell-free extract. Therefore, we have only assumed that the nonexchangeable level of drug in marrow is solely due to the binding of drug by dihydrofolate reductase. Since the same relationship between nonexchangeable levels of drug and the binding equivalence of dihydrofolate reductase has been established in our prior (12, 15–17) studies for mouse small intestine and liver and for various murine tumor cells, this would appear to be a reasonable assumption in the absence of any evidence showing tight binding by other proteins in any of these tissues. The value used for the drug-binding equivalence of the initial dihydrofolate reductase level in marrow was derived from the nonexchangeable level after a dose of methotrexate (12 mg/kg). It
should also be pointed out that the values estimated for non-exchangeable levels of drug (Charts 3 and 4) in both small intestine and marrow are 25 to 30% higher than the initial dihydrofolate reductase level at doses of methotrexate above 12 mg/kg. This result was similar to that observed in small intestine and tumor cells during our prior studies (14–18) and appears to be accounted for at least in part by binding of drug to newly synthesized dihydrofolate reductase. Protein synthesis in small intestine and marrow after different doses of methotrexate. In these experiments, we assessed drug-related effects on DNA synthesis by measuring effects on incorporation of m[H]dUrd administered as a short pulse (5 to 10 min) at varying time intervals after the drug was given. The results are given in Chart 5. Essentially complete inhibition of incorporation occurred by 60 min in both tissues after the administration s.c. of 12, 48, or 400 mg methotrexate per kg. At every dose studied, the onset of recovery of incorporation was much more rapid in marrow than in small intestine. However, the onset of recovery after each dose showed the same overall correlation with the duration of persistence of exchangeable levels of drug in each tissue. That is, as levels of drug approached the estimated nonexchangeable level, onset of recovery of m[H]dUrd incorporation was observed. Values for the time required for 50% recovery of incorporation in each tissue after each dose of methotrexate are summarized in Table 1. Although the absolute recovery times are not the same, the relative difference in recovery time among dosages was similar in each tissue. Also, at least at the 2 lower doses of methotrexate, the relative difference at each dosage between recovery time in each tissue was approximately the same. It should also be mentioned that these doses of methotrexate had no detectable effect on the total number or morphology of cells in marrow. However, a reduction in average weight of small intestine was observed beginning 24 hr after the administration of 400 mg methotrexate per kg.

DISCUSSION

The data presented document a markedly greater accumulation and persistence of methotrexate and a much slower onset of recovery of DNA synthesis in mouse small intestine as compared to marrow which appears to account for the greater sensitivity of the former to the cytotoxic effects of this folate analog. The correlation between the persistence of drug and the duration of inhibition of DNA synthesis observed in each tissue is similar to that reported (14, 16–18) from this laboratory earlier for mouse small intestine and various murine tumor cells. It was also of interest to note that, even though the absolute dose dependence for drug persistence required for a sustained inhibition of DNA synthesis in small intestine in this study is different than that required for the same duration of inhibition in our earlier (14, 16–18) studies, the relationship established in each case was the same.

The results of these and prior (14, 16–18) studies from our laboratory and elsewhere (2, 5) are consistent with differences in the cytotoxicity of methotrexate observed in these 3 tissues in the order, tumor ≫ small intestine ≥ marrow for responsive murine tumor models such as the L1210, P288, or P388 leukemias. Related evidence also reported (3) from our laboratory associated these pharmacokinetic differences between small intestine and tumor cells with differences in the ability of the carrier transport system shared (reviewed in Ref. 12) by methotrexate and natural folate coenzymes to maintain intracellular levels of methotrexate necessary to inhibit DNA synthesis in the face of rapidly falling plasma levels of drug. It is likely that the pharmacokinetics observed in marrow reflect to a great extent the properties of the corresponding cellular transport system.

The overall significance of these findings to folate analog pharmacology is severalfold. The findings establish the same
pharmacokinetic basis for differences in the recovery of these normal proliferative tissues following methotrexate treatment that was seen among various tumor cells. The degree of documentation provided by these and earlier (12—19) studies now allows us to propose with some degree of confidence that the property for accumulating and maintaining pharmacologically effective intracellular levels of folate analogs is differential among all proliferative tissues of this rodent and probably in all mammals. These findings also delineate a concept for the basis of selective action in the case of responsive tumor models which takes into consideration antiproliferative effects against tumor within the context of limiting effects against both major host renewal sites. In so doing, further validity is derived for an extrapolation of a concept elucidated in animal models to patients where antifolate effects in bone marrow are as serious (reviewed in Refs. 1 and 4) as in gastrointestinal tissue. The ultimate importance of these and our earlier (12—19) findings to the clinical use of antifolates remains to be determined.

However, it would seem that pharmacological studies of new folate analogs in murine tumor models which also take into consideration events in bone marrow would provide information of special relevance to the clinical development of more effective therapy with this class of agents.

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