Differential Cell Permeability and the Basis for Selective Activity of Methotrexate during Therapy of the L1210 Leukemia

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SUMMARY

The relationship of cell permeability phenomena to the selective activity of methotrexate (MTX) during therapy of the L1210 leukemia was examined. Drug uptake and loss in normal tissue (small intestine) and L1210 cells in the peritoneal cavity were measured following the administration of single s.c. doses of 0.03, 0.3, and 0.03 mg of MTX per kg. Within 1 hr after a dose of 3 mg/kg, drug levels in L1210 cells and small intestine were, respectively, four- and sixfold greater than the dihydrofolate reductase content. After 0.3 mg/kg, the initial rate of uptake in both cases was similar, but the intracellular level exceeded the enzyme content in only L1210 cells. After a dose of 0.03 mg/kg, uptake was almost nonexistent in small intestine but still appreciable in L1210 cells. Free MTX was lost from small intestine far more rapidly then from L1210 cells. After 3 mg/kg, drug levels in intestine fell to enzyme level in 3 hr, but in L1210 cells the fall required 18 to 20 hr. After a dose of 0.3 mg/kg, approximately 8 hr were required for the drug level in L1210 cells to reach enzyme level.

Following a dose of 3 mg of MTX per kg, administered i.p., initial uptake was more rapid and reached higher intracellular levels in both L1210 cells and small intestine when compared to that which occurred following the same dose given s.c. Drug also persists in L1210 cells in the peritoneal cavity, but not small intestine, for a somewhat longer period of time following i.p. administration. Uptake and loss of MTX following a dose of 3 mg/kg s.c. is the same in small intestine from normal and leukemic mice.

Incorporation of deoxyuridine-3H into DNA of both small intestine and L1210 cells was almost completely inhibited by 1 hr after a s.c. dose of 3 mg/kg. Resumption of incorporation required 4 hr in small intestine but 14 to 16 hr in L1210 cells. The results reveal a far greater persistence of free MTX and a more sustained metabolic effect in L1210 cells than in small intestine. This is attributed, at least in part, to a more effective influx of drug in L1210 cells at low plasma levels.

INTRODUCTION

The basis for the selective action of effective anticancer drugs has, for the most part, been difficult to elucidate. This is particularly true with the antifolates, a class of anticancer agents in clinical use for more than 2 decades (5). The antifolates are excellent inhibitors of dihydrofolate reductase from both tumor cells and normal tissue (1, 2, 12, 23, 32). The consequence of this inhibition for DNA synthesis probably accounts for the ultimate cytotoxic manifestation observed (17, 20). However, since both normal tissue and drug-sensitive tumor cells contain roughly the same amount of the "target" enzyme (1, 2, 9, 12, 23, 22, 23, 26, 32) and enzyme from responsive and unresponsive tumors are inhibited essentially to the same extent (1, 2, 12, 23, 26, 32), it is unlikely that the interaction of drug at this site can account for the selective effect seen during therapy. On the other hand, only therapeutically responsive tumors have been shown (3, 4, 6-8, 10, 18-20, 29-31) to possess an efficient mechanism for transporting and concentrating antifolates intracellularly. Although this correlation has provocative therapeutic implications, its relevance to selective activity is not at all clear, since normal cells, notably in small intestine, liver, kidney, and hematopoietic tissue, are also able to accumulate these drugs rapidly (19, 22, 24, 25, 27, 28).

In an effort to assess more clearly the role played by permeability in an actual therapeutic situation, we have made a direct examination of drug uptake in normal tissue and tumor cells following the administration of MTX to animals bearing the L1210 leukemia. Additional data on the incorporation of UdR-3H into DNA at different intracellular drug levels are also presented.

MATERIALS AND METHODS

L1210 cells (line V) were transplanted in C57BL/6 x DBA2 male mice as previously described (15). MTX was provided by Lederle Laboratories, Pearl River, N. Y. Three days prior to termination, drug was given and the L1210 cells were harvested from the peritoneal cavity of sacrificed animals by injecting 3 ml of cold 0.14 M NaCl-0.02 M sodium phosphate, pH 7.4, opening the cavity, and draining the cell suspension. If the ascitic fluid was hemorrhagic, the L1210 cells were washed twice with buffered 0.14 M NaCl-0.02 M sodium phosphate, pH 7.4, to lyse red blood cells. The L1210 cells were resuspended briefly in buffered 0.01 M NaCl-0.002 M sodium phosphate, pH 7.4, to lyse red blood cells. The L1210 cells were then washed twice with buffered 0.14 M NaCl-0.02 M sodium phosphate, pH 7.4, and drug was extracted by heating (30). After removal of the L1210 cells, the small intestine was surgically removed and placed in

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cold 0.14 M NaCl-0.02 M sodium phosphate, pH 7.4. The
organ was opened longitudinally, cleaned, homogenized,
and heated to extract drug in a manner described previously
(22). Small intestine from nonleukemic animals was also
removed and extracted in the same way. The drug content
in both extracts was determined by enzymatic assay (29).
Values are averages based on determinations with 2 to 4
animals. The dihydrofolate reductase content in unheated
extract from both normal and tumor cells was obtained by
titrination with antifolate (22, 25, 32). The incorporation of UdR-3H into DNA was determined
for at least 40 min in each case. Values given are average of
determinations on 3 to 5 animals.

RESULTS

MTX was administered s.c. to leukemic animals at 3
different doses: 3, 0.3, and 0.03 mg/kg. The lowest dose is
well below that required for minimum antileukemic activity
(13, 15). On a dosage schedule of every other day, 0.3
mg/kg would be expected to have a slight effect, whereas 3
mg/kg will achieve a maximum, or nearly maximum, an-
tileukemic effect [approximately 150% increase in average
survival time (13, 15)].
The uptake of MTX by leukemia cells in the peritoneal
cavity was compared to that which occurs in the small
intestine. This comparison was made because the crypt
epithelium of the small intestine appears to be the most
drug-sensitive normal tissue in mice (22). The s.c. route of
administration was originally selected, since it would proba-
ably not favor the initial uptake of MTX by L1210 cells in the
peritoneal cavity. The data obtained on uptake by these
cells, therefore, can reasonably be expected to be similar to
uptake by L1210 cells elsewhere in the animal. The
intracellular level of drug in L1210 cells and small intestine
at various times following s.c. administration to leukemic
animals is shown in Chart 1. At 0.3 and 3 mg/kg,
accumulation of drug was somewhat more rapid in small
intestine. Maximum levels of drug were attained in this
tissue in less than 1 hr, as compared to 2 hr in L1210 cells.
The maximum amount of drug accumulated after a dose of
3 mg/kg was about the same in both types of cells. When
related to drug equivalents of dihydrofolate reductase, the
maximum level in the small intestine was 6-fold greater but
only 4-fold greater in L1210 cells. The difference in free
MTX can be attributed to a somewhat higher level of
enzyme in L1210 cells. After a dose of 0.3 mg/kg, the level
of drug accumulated exceeded the enzyme content in L1210
cells, but not in small intestine. After the smallest amount
(0.03 mg/kg) of MTX was given, the uptake of drug in
L1210 cells, compared to small intestine, was much more
rapid and reached higher levels. In L1210 cells, drug
accumulation approached enzyme level in less than 1 hr but
only 5 or 10% of enzyme level in small intestine after 2 hr.
There is a dramatic difference (Chart 3) in the rate at
which free MTX was lost from L1210 cells and intestinal
surface. The content of drug in the small intestine was at
enzyme level within 3 hr after 3 mg/kg. By comparison,
drug content of the L1210 cells did not reach enzyme level
until 18 to 20 hr after the administration of the same dose.
Even after giving 0.3 mg/kg, drug content of L1210 cells
did not reach enzyme level until nearly 8 hr. It is also noted that
loss of drug from dihydrofolate reductase in both L1210
cells and small intestine occurred to a significant extent,
although slowly, within the period of the experiment. This
result is in agreement with prior observations made on the
rate of drug loss from dihydrofolate reductase in small
intestine (25).
The level of uptake of MTX by L1210 cells and small
intestine was also examined after the i.p. injection of drug.
This route of injection results in only a slight, if any,
increase in therapeutic effect when compared to the s.c.
route (D. J. Hutchison, private communication). However,
a more rapid initial uptake of drug by L1210 cells in the
peritoneal cavity would be expected. A comparison of the
results obtained after 3 mg/kg were administered by each
route is shown in Chart 2. As anticipated, MTX was con-
centrated in L1210 cells to much higher levels following i.p.
injection. The intracellular level in 1 hr was 15 times greater
than the dihydrofolate reductase content. The accumula-

![Chart 1. The uptake and loss of MTX in L1210 cells and small intestine after s.c. administration of different amounts to leukemic mice.](image1)

![Chart 2. The uptake and loss of MTX in L1210 cells and small intestine after s.c. or i.p. administration of 3 mg/kg to leukemic mice.](image2)
tion in L1210 cells, following the same dose given s.c., is also shown for comparison. Although very high levels were achieved by the i.p. route, they were found to diminish rapidly. Even so, after 3 mg/kg, the intracellular level was still about twice the enzyme content in 20 hr.

The initial uptake of MTX by small intestine was also higher following an i.p. dose, although not to the same extent seen in L1210 cells. After 3 mg/kg, drug was concentrated to a level about 30% higher than that attained by the s.c. route. Despite the difference in the initial uptake of MTX, intracellular levels were down to enzyme level in about the same time.

The greater persistence of drug in the L1210 cells in the peritoneal cavity could explain the better therapeutic effect of an i.p. dose. Although the survival of mice bearing this leukemia is probably related to the pathological changes that occur during the progression of the leukemia, the degree of L1210 cell proliferation in the peritoneal cavity might also be of some importance.

In a related experiment, the uptake and subsequent loss of MTX in small intestine was also studied in nonleukemic animals. A comparison with “leukemic” intestine was of special interest, since it has been previously shown (14) that a marked diminution in the size of the small intestine occurs during the development of the leukemia. One might also anticipate a corresponding change in metabolic disposition which could alter drug distribution in this tissue. The results obtained after a dose of 3 mg/kg was given is shown in Chart 3. The rate and amount of drug uptake and the loss of drug with time was quite similar in small intestine from normal and leukemic animals. The content of dihydrofolate reductase was also found to be about the same in both tissues.

A direct indication of the metabolic consequences of various intracellular drug levels was obtained by measuring UdR-3H incorporation into DNA after the administration of 3 mg of MTX per kg. The data on the relative extent of UdR-3H incorporation are given in Charts 4 and 5. The values given represent the percentage of incorporation obtained at each interval when compared to the amount of incorporation obtained in a group of untreated controls.

Chart 3. The uptake and loss of MTX in small intestine from normal and leukemic mice after s.c. administration of 3 mg/kg.

Chart 4. Intracellular MTX levels and the incorporation of UdR-3H into DNA of small intestine at varying intervals following the administration of 3 mg/kg to leukemic mice. The level of radioactivity incorporated in controls was 6000 to 8000 cpm/g of intestine.

Chart 5. Intracellular MTX levels and the incorporation of UdR-3H into DNA of L1210 cells at varying intervals following the administration of 3 mg/kg to leukemic mice. The level of radioactivity incorporated in controls was 3 to 4 × 10^4 cpm/g of cells.

The level of MTX in L1210 cells and small intestine at various intervals is also shown. Cessation of UdR-3H incorporation into the DNA of both L1210 cells and intestine is nearly complete in 1 hr. This is in agreement with the amount of drug uptake obtained in both cases. Resumption of UdR-3H incorporation occurred within 4 hr in small intestine (Chart 4). At this point, the level of MTX was already below enzyme level. Recovery of UdR-3H incorporation continues and approaches maximum in 12 hr. Further recovery after this point occurred more slowly. This seems commensurate with the very gradual loss of drug from enzyme also observed. These results on UdR-3H incorporation into DNA of small intestine of leukemic animals at different drug levels are very similar to that reported for small intestine in normal animals (20, 23).
Resumption of incorporation of UdR-3H into the DNA of L1210 cells occurred much later then in small intestine. No incorporation was detectable before 14 hr after the administration of 3 mg/kg (Chart 5). However, recovery occurred steadily thereafter, so that incorporation was at 40% of the maximum rate at 20 hr. Some discrepancy was observed in L1210 cells relating to the degree of UdR-3H incorporation at low-intracellular drug levels. Appreciable incorporation of label occurred when the drug level was still above enzyme content. This is most likely due to the partial stoichiometry of the dihydrofolate reductase-MTX interaction. Inhibition of L1210 enzyme activity in vitro has been shown to have a titration relationship to drug concentration only to about 80% of the total number of drug binding sites (11, 16). Also, complete inhibition of UdR-3H incorporation following uptake by L1210 cells in vitro does not occur until the intracellular drug level is nearly twice the enzyme level (F. M. Sirotnak and R. C. Donsbach, unpublished results).

DISCUSSION

The data clearly reveal a far greater persistence of MTX in L1210 cells when compared to small intestine. Moreover, the degree of persistence in L1210 cells at different dosage correlates quite well with the antileukemic effect obtained (Refs. 13 and 15; D. J. Hutchison, private communication). In understanding the reason for this difference in drug loss between the leukemia cells and normal tissue, the data obtained on the uptake of MTX after the administration of 0.30 mg/kg are important. At this low concentration, the rate of uptake within the 1st 0.5 hr in L1210 cells is about 10 times higher than that seen in small intestine. Since levels of MTX in plasma fall very rapidly soon after administration (22, 24, 25), this greater ability of L1210 cells to accumulate drug at low concentration becomes a critical factor in maintaining intracellular levels above the dihydrofolate reductase content.

Assuming only minimum differences related to drug distribution within body fluids, the approximate rate of drug uptake by L1210 cells and small intestine obtained within the 1st hr after the administration of varying amounts would suggest a mechanism for concentrating drug in each case, which is about equal in capacity but more efficient in L1210 cells. A greater affinity of MTX for the mechanism in L1210 cells could account for a more efficient operation at low-plasma concentrations. Studies on uptake by L1210 cells in vitro provide (7, 18, 29-31) ample evidence for an exceptionally high affinity of the carrier-transport system for MTX.

Overall, the results provide evidence for a basis for selective activity of MTX in the L1210 cell system, which is at least partially related to differences in cell permeability between target tumor cells and normal host tissue. They do not, by themselves, exclude the possibility that other physiological differences may exist as well not only in this system but in other host-tumor systems and patients with responsive leukemias.

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