Modes of Uptake of Methotrexate by Normal and Leukemic Human Leukocytes in Vitro and Their Relation to Drug Response

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SUMMARY

Characteristics of Methotrexate transport by normal and leukemic human leukocytes were measured in vitro. At high external drug levels (≥20 μM) drug uptake by all cell types was temperature-insensitive, nonsaturable, and nonconcentrative. The steady-state cell/medium drug distribution ratio was about 0.03. During clinical treatment with Methotrexate, plasma drug levels generally reach 0.2 μM. At this lower drug level: (a) A temperature-sensitive and saturable mode of transport was demonstrable in all cell types except small lymphocytes. In normal granulocytes the steady-state drug distribution ratio reached 0.2. (b) Leukemic cell types showed a wide variation in capacity for uptake of the drug, and an association was seen between this capacity and clinical response to the drug.

INTRODUCTION

Methotrexate (Amethopterin, 4-amino-N10-methylpteroyl-glutamic acid) is a folic acid antagonist used in the treatment of human leukemias; the drug can also prolong survival of animals bearing certain neoplasms. Resistance to the drug in human (2, 3, 5) and animal (10, 12, 16) leukemias has been associated with high cellular levels of dihydrofolate reductase, an enzyme inhibited by the drug (1, 42, 43). However, many amethopterin-insensitive human (33) and animal (35) leukemias did not contain reductase levels which were significantly higher than those found in drug-sensitive neoplasms. Therefore, the role of Methotrexate transport was considered as a possible factor in drug response. Evidence showing mediated uptake of Methotrexate by mammalian cell types has been reported (22, 23, 37). Uptake of the drug was impaired in drug-resistant animal leukemias (11, 25, 44) and drug-resistant microorganisms (28, 39, 46). We have therefore studied the nature of Methotrexate transport in normal and leukemic human leukocytes. Preliminary reports of these data have been presented (24, 25).

MATERIALS AND METHODS

Isolation and Treatment of Leukocytes. Human blood was collected, using Versene or citrate as an anticoagulant, and the leukocytes were isolated as described by Fallon et al. (9). Dextran sedimentation of erythrocytes was followed by osmotic lysis to eliminate the remaining red cells. Small lymphocytes were separated from granulocytes by the procedure of Ventzke et al. (41). This involved centrifugation of leukocyte suspensions in long tubes for 15 minutes at 100–150 × g. The top layer of cells, predominately small lymphocytes, was collected. If present in significant numbers (>10%), contaminating granulocytes were removed on columns of siliconized glass beads (30). The bottom layer of cells consisted of 85–90% granulocytes contaminated mainly with large lymphocytes. The latter could be isolated by removing granulocytes on glass bead columns. Leukocytes from patients with leukemia were used after isolation by the Fallon method. If significant numbers of granulocytes (>20%) were present, these were removed on glass bead columns (31). Wright-stained slides were made of all cell preparations to determine relative proportions of each cell type. In data presented here, cells from patients with chronic myelogenous leukemia were mainly (70–95%) immature granulocytes, and those from acute leukemias were 80–100% blast forms. Viability was estimated by ability of cells to exclude 0.025% trypan blue. Less than 10% of the cells in preparations used took up the dye.

Leukocytes were suspended in an incubation medium (23) containing one part of dialyzed calf serum (obtained from Grand Island Biological Co., Grand Island, New York) and three parts of buffer [62 mM TES buffer (13) at pH 7.2, 65 mM NaCl, 15 mM KCl, and 8 mM CaCl₂]. The incubation of granulocytes, in the absence of serum, resulted in cell clumping and an increase in the number of cells permeable to trypan blue. Addition of streptokinase-streptodornase (9, 36) to prevent cell clumping was not necessary when serum was included in the medium. Labeled Methotrexate (2–5 μl of stock solution) was added to 150–μl aliquots of cell suspensions (8–15 mg of cells wet weight), which were then incubated in stoppered 10 × 30 mm siliconized glass tubes, with gentle shaking, for 1–60 minutes. The incubations were terminated by chilling the suspensions and adding 750 μl of ice-cold buffer. The cells were collected by centrifugation at 150 × g for 30 seconds. A model 5500 microcentrifuge was used (Microchemical Specialties Co., Berkeley, California). A portion of the supernatant fluid was saved for determination of the Methotrexate-3H concentration. The cell pellets were washed by suspending in buffer at 0°C.
followed by centrifugation. In some experiments, the rate of loss of labeled drug was measured by suspending the previously loaded cells in 250 μl of fresh medium and incubating them for varying times at 0–37°C. The cells were collected as before. To extract and measure accumulated Methotrexate, the washed cells were suspended in 250 μl of 0.01 N acetic acid and heated to 60°C for 10 minutes (47). After centrifugation, a 200 μl portion of the supernatant fluid was taken for measurement of radioactivity by liquid scintillation techniques. The cell debris contained no radioactivity.

Calculation of Drug Distribution Ratios. The drug distribution ratio of Methotrexate achieved after incubations was determined by measurement of intracellular and extracellular levels of radioactivity; this is reported in terms of moles of drug per gm of cells divided by moles of drug per ml of medium. Results, unless otherwise stated, are expressed as the average of at least three determinations. The range observed generally differed from average values by no more than ±10%.

Purification and Standardization of Methotrexate Solutions. Methotrexate-3'-5'-3H (2-9 c/mmole, purchased from the Nuclear-Chicago Corp.) and nonradioactive Methotrexate (supplied by Dr. J. M. Rueggsegger, Lederle Laboratories) were purified by descending chromatography on Whatman No. 1 paper, using 0.01 M sodium phosphate at pH 7.5 as the solvent. The UV-absorbing region at RF = 0.6–0.7 was cut from the paper and eluted with water. The water was removed by lyophilization, and the purified drug was dissolved in water to a concentration of 20 μM. Larger quantities of nonlabeled drug were purified as recommended by Johns (21) or by Loo (27). The concentration of stock solutions of labeled drug was determined by titration against dihydrofolate reductase obtained from rat liver (32). Purified nonlabeled Methotrexate was used as the standard.

RESULTS

General Observations. The external Methotrexate level used for most experiments was 0.2 μM, which corresponded to the plasma levels generally found during clinical treatment with the drug (18).

Washing the cell pellets with buffer at 0°C after incubations with labeled drug was estimated to remove 99% of the extracellular drug. Studies using labeled inulin, which is excluded from leukocytes (36, 47) showed that a cell/medium distribution ratio of no more than 0.005 could be attributed to contamination of cells by extracellular radioactivity not removed by washing. The inulin content of packed cell pellets, which were not washed, showed that 30–40% of the pellet space was extracellular.

No significant loss of intracellular drug occurred during the washing procedure used here. The intracellular Methotrexate accumulated by leukocytes is therefore not sensitive to "cold shock" (40), since washing of incubated cells at either the incubation temperature or at 0°C gave identical results when the drug distribution ratio was calculated. Furthermore, the loss of Methotrexate from previously loaded cells upon resuspension in medium at 0°C was negligible (Chart 4).

The sodium, potassium, and calcium levels in the buffer used here permitted optimal uptake of model amino acids by human leukocytes (47). Binding of Methotrexate to serum proteins has been reported (18) and, probably for this reason, addition of serum to the medium (25% v/v) decreased the rate of Methotrexate uptake by leukocytes by about 20%. Omission of the calcium from the medium stimulated the rate of Methotrexate uptake by 10–50%, as did replacement of a portion of the NaCl of the medium with 20 mM Versene or sodium phosphate. Since addition of phosphate to the medium resulted in clumping of cell preparations, this ion was usually omitted. Addition of 1–5 mM MgSO4, 1–10 mM glucose, or incubation of cells under anaerobic conditions did not alter the rate of drug uptake nor did addition of 10−3 M dinitrophenol.

Often, preparations of lymphocytes or leukemic leukocytes did not require the siliconized glass bead purification procedure. Data obtained with these cells did not differ significantly from results on cells from the same preparation which were given the additional treatment. We concluded that the transport of Methotrexate was not affected by passage of cells through columns of siliconized beads.

Uptake of Methotrexate by Normal Leukocyte Types. The effect of variation of incubation temperature on the time course of Methotrexate uptake by normal granulocytes and small lymphocytes is shown in Chart 1. When the drug level in the medium was 0.2 μM, influx into granulocytes, but not lymphocytes, was highly temperature-sensitive. An apparently rapid phase of drug uptake, estimated by extrapolation of the

![Chart 1. Drug distribution ratio of Methotrexate after incubation of normal cell types in medium containing 0.2 μM drug. The results of a typical experiment are shown; repeated measurements on different cell preparations did not differ from these data by more than ±10%.](cancerres.aacrjournals.org)
uptake data to zero time, was similar in extent in both cell types.

Evidence indicating saturability of Methotrexate uptake by granulocytes, but not by small lymphocytes, is shown in Chart 2. The drug distribution ratio achieved by granulocytes after a 30-minute incubation fell from 0.2 to 0.03, when drug level in the medium was raised from 0.2 to 200 μM. Methotrexate uptake by small lymphocytes was essentially nonsaturable; the drug distribution ratio remained at about 0.025 regardless of the extracellular drug level. Data on normal large lymphocytes are scarce because of difficulties in isolation in large quantities, but it appears that these cells have a greater capacity for Methotrexate uptake than do small lymphocytes (Chart 5).

The results of a more detailed study of the effect of temperature variation on the initial rate of drug uptake by leukocytes is shown in Chart 3. The rates were obtained from data taken over 5-minute intervals, as shown in Chart 1. Uptake of the drug by granulocytes was especially temperature-sensitive over the 27-37°C range. The time course of the drug loss from leukocytes, shown in Chart 4, was measured after loading leukocytes by incubation for 15 minutes at 37°C in medium containing 0.2 μM Methotrexate. Again, granulocytes showed the most striking dependence of rate loss of Methotrexate upon incubation temperature.

**Uptake of Methotrexate by Leukemic Leukocytes.** The capacities of normal and leukemic cell types for Methotrexate transport are compared in Chart 5. Cells were incubated with 0.2 μM drug for 15 minutes at 37°C. Uptake of Methotrexate during 15-minute incubations of all of these cell preparations was decreased to 4–6 μmoles/kg cells by lowering the temperature to 0°C. This was judged to represent the contribution to drug accumulation of a temperature-insensitive uptake mode. The routine 15-minute incubation period at 37°C permitted observations to be made during the linear period of drug uptake. This was verified with about two-thirds of the different cell preparations, e.g., see Chart 6. Measurements were generally made at this single time point because of the limited number of cells available and because clumping of leukemic cell preparations occasionally occurred after 20–25 minutes of incubation.
Drug-sensitive patients, listed in Chart 5, were those who later responded favorably to Methotrexate therapy, as shown by a fall in both the total number of circulating leukocytes and a shift to a more normal cell population. This will be considered more fully later. Chronic lymphocytic leukemias were classified as having an unknown response to Methotrexate, although a response would be unexpected. These patients were not treated with the drug since the lack of response of this disease to Methotrexate is well known, and formed the basis for omission of this agent from their treatment. Methotrexate has rarely been used in treatment of chronic myelogenous leukemias, but the data shown in Chart 5 suggest the possible usefulness of the drug in selected patients.

A more detailed study of two lymphoblast preparations (designated a and b in Charts 5, 6 and 7) showed that Methotrexate-sensitive leukemia cells had a marked capacity for uptake of the drug via a temperature-sensitive and saturable process. When the Methotrexate level in the medium was 0.2 \( \mu M \), a drug-distribution ratio of 0.17 was achieved after a one-hour incubation at 37°C. In contrast, a drug-distribution ratio of only 0.05 could be achieved with lymphoblasts from the patient with a Methotrexate-resistant leukemia. Drug uptake by both cell preparations was similar when the extracellular drug level was elevated to 200 \( \mu M \) or when low incubation temperatures were employed. The data described here are typical of results obtained using thirty different preparations of myelogenous or acute lymphocytic leukemia cell types. At drug levels of 20–200 \( \mu M \) or at low temperatures, the distribution ratios were 0.01–0.02; higher distribution ratios were reached only at or near 37°C and at drug levels considerably below 20 \( \mu M \).

**DISCUSSION**

The most interesting implication of this work is the suggestion of correlation between capacity for Methotrexate uptake *in vitro* by isolated blast cells from patients with acute leukemia and responsiveness of the patients to the drug. This correlation was demonstrable at 37°C if extracellular drug levels were sufficiently low, so that an easily saturable process could be measured. In the present study, a drug level of 0.2 \( \mu M \) was generally used. This minimized the contribution to drug uptake of a nonsaturable transport process. Since the Methotrexate level generally found in blood during clinical therapy...
with the drug is about 0.2 μM (18), the use of this low level of drug for routine studies seems justifiable. In related studies, we have demonstrated the presence of a saturable process of Methotrexate transport in animal leukemia cells (22) which had not been observed when high drug levels were employed (20).

Among the normal human leukocyte types, transport of Methotrexate by small lymphocytes was nonconcentrative; the steady-state drug distribution ratio of 0.03 was independent of incubation temperature or of the extracellular drug level (Charts 1–3). Methotrexate was also poorly accumulated by certain animal leukemia cells (15). The difficulty in penetration of cells by this hydrophilic drug molecule, with a charge of −2 at pH 7, was not thought surprising (15). We found (data not shown here) that another similarly charged ion, sulfate, was excluded from all leukocyte types.

Normal granulocytes showed a type of Methotrexate transport not observed with small lymphocytes. When the extracellular drug level was sufficiently low (0.2–2 μM), a mode of drug uptake could be demonstrated which, although nonconcentrative, was both temperature-sensitive (Charts 1, 3) and saturable (Chart 2). From the data of Chart 2, and other experiments not reported here, this process appears to have a K_m of 1–2 μM. The steady-state drug distribution ratio attained by granulocytes was 0.2, which corresponds to the value predicted by Jacquez (20) for equilibrium distribution of the drug in Ehrlich carcinoma cells. The foregoing data have suggested to us (23) that granulocytes have the capacity for "facilitated diffusion" of Methotrexate by a saturable and temperature-sensitive process. The temperature-insensitive, and apparently nonsaturable, mode of Methotrexate uptake by granulocytes, demonstrable at low temperature (Charts 1, 3) and at high drug levels (Chart 2), may represent a different process, possibly simple diffusion. Simultaneous facilitated and nonfacilitated uptake of Methotrexate by a microorganism have been characterized by Sirotnak et al. (38, 39). The data of Chart 1 suggest the possibility of yet a third mode of drug uptake, an apparently instantaneous binding. A three-mode uptake of the model substrate benzylamine by Ehrlich carcinoma cells has been reported (7). Incomplete removal of extracellular radioactivity from cell pellets, or drug freely entering a portion of nonviable cells (20), could also account for this apparently instantaneous binding.

The uptake of Methotrexate by granulocytes was shown by us elsewhere (17) to have the type of structural specificity usually associated with a mediated transport process. Certain drug analogs (folinic acid, aminopterin, dichloroamethopterin, and N^{10}-ethylaminopterin, but not folic acid) could competitively inhibit uptake of Methotrexate by granulocytes. It is noteworthy that Rubin et al. (36) found that dichloroamethopterin, but not folinic or folic acids, inhibited uptake of Methotrexate by rabbit kidney slices; the L1210 and the Ehrlich ascitic tumors exhibited different specificities (22).

When Methotrexate transport by leukemic cell types was studied, chronic lymphocytic leukemia cells closely resembled

![Chart 6](chart6.png)

**Chart 6.** Drug distribution ratio of Methotrexate after incubation of two different lymphoblast preparations (patients a and b of Chart 5) in medium containing specified drug concentrations at the temperature indicated.

![Chart 7](chart7.png)

**Chart 7.** Initial rate of Methotrexate uptake from medium containing 0.2 μM drug by two different lymphoblast preparations, patients a and b of Chart 5.
normal small lymphocytes in their handling of Methotrexate (Chart 5). In other experiments, not shown here, data of Charts 1-4, obtained by using normal small lymphocytes, could be duplicated with the lymphocytes from patients with chronic lymphocytic leukemia. But the capacity for uptake of Methotrexate by lymphoblasts, myeloblasts, and cells of the myelocyte-promyelocyte series varied widely at 37°C with drug levels of 0.2 µM. At high levels, or at low temperatures, the variation in drug uptake among different leukemic cell preparations disappeared. Hence, the variation in Methotrexate uptake by leukemic cells is believed to reflect differences in capacity of cells for a saturable and temperature-sensitive mode of transport (Charts 6, 7).

These studies were all carried out in an incubation medium which provided suitable conditions for observation of uptake by leukocytes (23). Hakala (15) found that calcium ion enhanced Methotrexate uptake by Sarcoma 180 cells, but we found that absence of calcium slightly promoted Methotrexate uptake either by Li210 cells (22) or, in the present study, by human leukocyte types. Calcium (8 mM) was included in our media because it promoted cell stability during incubation. Verseen and phosphate enhanced the rate of Methotrexate uptake, probably because of the ability of these agents to bind calcium. In contrast to data shown here, Rubin et al. (37) found that phosphate (16 mM), calcium (2.5 mM) and magnesium (1 mM) were all necessary for optimal uptake of Methotrexate by rabbit kidney slices.

The nature of Methotrexate exodus from leukocytes has not been fully described and more work is needed to clarify the role of this process in net drug accumulation. Loss of Methotrexate from leukocytes was shown here to be temperature sensitive (Chart 4), suggesting the possibility of an active exit process, such as that found in Sarcoma 180 cells (14, 15). Cellular levels of dihydrofolate reductase, an enzyme which tightly binds Methotrexate (42), are too low to account for more than a small portion of the drug accumulated by normal leukocytes and chronic lymphocytic leukemia lymphocytes (33). Since enzyme-bound drug is not lost upon washing cells (34), the data of Chart 4 indicate that granulocytes have a capacity to take up Methotrexate in excess of the level required to inhibit dihydrofolate reductase. In other types of leukemic cells, dihydrofolate reductase levels were greatly elevated (1, 4, 34, 45) and could therefore significantly contribute to net Methotrexate accumulation (23, 25). However, the initial rate of Methotrexate uptake was not necessarily related to the level of this enzyme. For example, Methotrexate uptake by lymphoblast preparation b, Chart 5, was slower than uptake of the drug by normal lymphocytes, although the lymphoblasts contained a 25-fold higher level of dihydrofolate reductase than did normal granulocytes.

In related studies, we found that accumulation of Methotrexate by animal leukemias selected for resistance to the drug was 4-fold less than accumulation by parent cell lines (26), although resistance was accompanied by a 5–10 fold increase in levels of dihydrofolate reductase (35). Different rates of Methotrexate uptake were found in two animal leukemias which had almost identical levels of dihydrofolate reductase (22).

In contrast to the animal leukemia situation, it is extremely difficult to control the many variables involved in the clinical treatment of human leukemia. Hence, one must be conservative in interpreting the statistical relation found between transport and clinical response shown in Chart 5. Resistance in human leukemia, as used in this report, refers to inability of the drug to induce partial marrow remissions as defined by the Acute Leukemia Cooperative Group B of the National Cancer Institute (6). Methotrexate was given alone or in combination with 1 mg/kg of oral prednisone. The Methotrexate dose was orally or parenterally administered over a period of four to eight weeks and was sufficient to cause maximum tolerated gastrointestinal toxicity. Unless otherwise stated, the resistance observed was thus “natural,” as opposed to that which is acquired or drug-induced. Within these limits, and those imposed by the fact that in vitro studies must be done under nonphysiologic conditions, the data reported here appear highly suggestive of a relationship in which uptake of Methotrexate was poorer in the patients who failed to respond to the drug. In addition normal or leukemic small lymphocytes, classes of human cells which are resistant to Methotrexate, showed relatively poor capacity for uptake of the drug. The finding that granulocytes show a capacity for Methotrexate uptake not shared by small lymphocytes, as well as the more rapid rate of cellular turnover of granulocytes in the blood, might help to explain reports that administration of the drug to normal individuals leads to a fall in the granulocyte count at levels of drug which do not depress the lymphocyte levels (8, 19, 29).

These observations suggest that the relative capacity of leukemic leukocytes for an apparently mediated mode of Methotrexate transport in vitro showed a variation which was related to drug response. Further work will be necessary to determine whether measurements of drug uptake in vitro by leukemic cells will be a useful predictive index of responsiveness of patients with leukemia to Methotrexate.

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