Establishment of Methotrexate-resistant Human Acute Lymphoblastic Leukemia Cells in Culture and Effects of Folate Antagonists

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ABSTRACT

A human acute lymphoblastic T-cell line, MOLT-3, was fed with Roswell Park Memorial Institute Medium 1640-10% fetal bovine serum-antibiotics, containing increasing concentrations of methotrexate (MTX). After 10 months of feeding, the cells became resistant to $10^{-7}$ M MTX; resistance was not reversed when the cells were placed in the original MTX-free medium. At $10^{-7}$ M MTX, the concentration which produced complete inhibition of the parent MOLT-3 cell growth, the resistant cells were not inhibited at all. On a 50% inhibitory concentration basis, the resistant cells were approximately 30-fold more resistant to MTX. The parent MOLT-3 and the resistant line had the same doubling time of approximately 36 hr. There were no differences in light microscopic morphology. MOLT-3 produced loose colonies on 0.5% agar enriched with fetal bovine serum, whereas the colonies of the resistant line were tightly packed. The development of resistance was accompanied by a 4- to 5-fold decrease in $[^{3}H]$MTX transport (MOLT-3/MTX,). Kinetic analysis of MTX uptake showed that the resistant subline did not have an altered $K_{m}$ for MTX (6.6 /IM) but had a decreased $V_{max}$ of about 20% of the parent cell line. These data suggest that either the number of folate transport sites or the turnover rate of these sites has been reduced in the MTX-resistant cell line. Dihydrofolate reductase was only minimally elevated in the resistant cell line. The MTX-resistant cell line was cross-resistant to dichloromethotrexate. The sensitivity of the resistant line to the substituted 2,4-diaminoquinazoline and pyrimidine compounds, 2,4-diamino-5-methyl-6-[(3',4',5'-trimethoxyanilino) methyl] quinazoline (JB-11) and 2,4-diamino-5(3',4'-dichlorophenyl)-6-methylpyrimidine, increased more than 3-fold. While leucovorin equally reversed the MTX effects on the parent and resistant cells, leucovorin reversal of 2,4-diamino-5-methyl-6-[(3',4',5'-trimethoxyanilino) methyl] quinazoline and 2,4-diamino-5(3',4'-dichlorophenyl)-6-methylpyrimidine effects was limited only to the parent cell line. 2,4-diamino-5-methyl-6-[(3',4',5'-trimethoxyanilino) methyl] quinazoline or 2,4-diamino-5(3',4'-dichlorophenyl)-6-methylpyrimidine plus leucovorin might prove to be unique in treating patients with acute lymphoblastic leukemia when the leukemic cells develop transport resistance to MTX.

INTRODUCTION

The development of resistance to MTX$^{4}$ has been studied in animal and human tumors (see review, Refs. 1 and 4). An elevated level of the target enzyme, dihydrofolate reductase (3, 5), and/or impaired membrane transport of drug (21) have been shown to be major causes of resistance in human tumors. In order to overcome the MTX resistance, 2 approaches can be considered: (a) the use of high-dose MTX with LV rescue where a significant amount of MTX can enter cells by passive diffusion (8, 29) rather than through the classical saturable temperature-sensitive process of active transport (15, 29); (b) the use of folate antagonists which differ from MTX for their action.

MATERIALS AND METHODS

The cell line used was of human acute lymphoblastic leukemia, MOLT-3, with T-cell characteristics (17). It was maintained as a suspension in culture flasks (Falcon Plastics, Oxnard, Calif.) containing RPMI 1640 Medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% FBS (Grand Island Biological Co.) and antibiotics (22, 28). Cells were fed with fresh culture medium 3 times a week. Effects of folate antagonists on cell growth were determined by growth inhibition assay, as described previously (28). The cells were in an exponential growth phase and had over 90% cell viability. Drugs tested in growth inhibition assays were MTX and LV (Lederle Laboratories, Pearl River, N.Y.), DCM (obtained from the National Cancer Institute, Bethesda, Md.), TMQ (JB-11; obtained from Dr. J. Plowman, Drug Evaluation Research, the National Cancer Institute), DDMP (Burroughs-Wellcome Co., Research Triangle Park, N. C.; Lot 58167, obtained through the courtesy of Dr. S. Waxman), 5-fluorouracil (Roche Laboratories, Nutley, N. J.), 1-β-D-arabinofuranosylcytosine (The Upjohn Co., Kalamazoo, Mich.), Escherichia coli asparaginase (Merck, Sharp & Dohme, West Point, Pa.), cisplatin (Bristol Laboratories, Syracuse, N. Y.), and daunorubicin (obtained from the National Cancer Institute).

MTX and DCM were dissolved in 0.9% NaCl solution. DMPM was initially dissolved in a small quantity of 0.1 M HCl, and TMQ was dissolved in pure ethanol before being further diluted with 0.9% NaCl solution. Preliminary studies showed that the vehicles used (similarly...
diluted HCl or ethanol in the culture medium) did not influence the cell growth. For all experiments, serial drug solutions were freshly prepared just before use.

Ten-ml cell suspensions of the parent cell line and subline were prepared in individual culture tubes (no. 3033; Falcon) at an initial cell density of 1.5 × 10⁶/ml, to which 0.1 ml of drug solutions at graded concentrations was added. The cells were incubated at 37°C. Three days later, the viable cell number was counted by the trypan blue dye exclusion method; dose-response curves were obtained by calculating the percentage of viable cells in drug-treated tubes as compared to those in control tubes without drugs. To determine the reversal effects of LV against folate antagonists, equimolar concentrations of LV (0.1 ml) and each drug (0.1 ml) at graded concentrations were added successively to 10 ml of the cell suspension. After 3 days of incubation at 37°C, the protective effect of LV was determined by comparing dose-response curves with and without LV. All experiments were carried out in triplicate and repeated at least twice.

For the morphological study, cells were examined under light microscopy after Wright-Giemsa staining. Cells were also plated on replica plates (16) containing 0.5% agar in Roswell Park Memorial Institute Medium 1640 enriched with 10% FBS and incubated at 37°C in 5% CO₂ in humidified air. Cell volumes of the parent and resistant cell lines were measured by the Coulter Counter Channelizer (Coulter Electronics, Hialeah, Fla.) and were calibrated using standard spheres with diameters of 5.02, 10.02, and 14.76 μm (Coulter).

The second method was MTX-binding capacity assay (14). For this study, [3H]MTX (6 to 12 Ci/nmol; Amersham) was purified on DEAE-cellulose (DE52, Whatman). After preincubation of the cells for 15 min at 37°C, [3H]MTX or 5-[14C]-MeFH₄ (58 pCi/ml) was added. The reaction mixtures were centrifuged at 25,000 x g for 10 min. Aliquots of 100 μl of the supernatant were added to 5 ml of ACS (Amersham) and analyzed for radioactivity.

RESULTS

Development of the MTX-resistant Cell Line, MOLT-3/MTX. A resistant subline was developed by growing MOLT-3 cells in the continuous presence of MTX. The initial MTX concentration used, 5 × 10⁻⁹ M, was raised by 1.5- to 2-fold successively. With increase in MTX concentration, the cell growth rate was temporarily decreased; it took 2 to 4 months until the growth rate returned to that of the parent cell. The process was then repeated until the MTX concentration reached 10⁻⁷ M. At this stage, the MTX-resistant cells were fed in the same concentration of MTX for 3 months and then returned to the original MTX-free medium and subjected to characterization studies. The parent line and the resistant subline had the same doubling time of approximately 36 hr. There were no differences in morphology. On the replica plate, the parent MOLT-3 produced loose colonies, whereas the colonies of the resistant line were tightly packed. The cell volume of the parent and resistant cells was equal. This subline seemed to be a stable mutant. Thus, during the 16-month observation period, MTX resistance of the subline in the MTX-free medium had not changed. Inhibitory effects of MTX on the parent MOLT-3 and the resistant MOLT-3/MTX cells determined by cell growth inhibition assay are illustrated in Chart 1. At the MTX concentration of 10⁻⁷ M, the viable cell growths of the parent cells were completely inhibited, whereas the growth of the resistant cells was uninhibited. The ID₅₀ of MTX were 10⁻⁶ M for the parent cells and 3 × 10⁻⁶ M for the resistant cells. Thus, on an ID₅₀ basis, the MOLT-3/MTX cells were approximately 30-fold more resistant to MTX than were the parent cells.

The cells were washed 3 times by centrifugation for 3 sec in a Beckman Microfuge B, and then the cell pellet was dissolved in 0.5 ml of NCS (Amersham) overnight at 37°C. The samples were counted in a Beckman Model 250 liquid scintillation counter at 30% counting efficiency. The results were calculated as a net uptake as measured by the total uptake minus the diffusion value at 0°. Efflux was similarly measured after diluting an aliquot of the cells into 20-fold excess drug-free medium.
Dihydrofolate Reductase Activity and Radioactive MTX Uptake. To clarify the mechanism of MTX resistance of the subline, dihydrofolate reductase activity and intracellular transport of $[^3H]$MTX were compared with the parent cell line. The results of the enzyme assay are summarized in Table 1. At a MTX concentration of $5 \times 10^{-9}$ M, extracts from both cells exhibited tight-binding kinetics; i.e., both enzymes were inhibited stoichiometrically when the cell supernatant was preincubated with MTX prior to addition of the folate substrate. Either the amount of dihydrofolate reductase ($[^3H]$MTX assay) or the functional activity ($[^3H]$dihydrofolate assay) was only minimally elevated in the resistant cells. The 30-fold differences in the sensitivity between parent and resistant cells cannot be accounted for on the basis of increased dihydrofolate reductase alone.

Results of the MTX transport in EBSS are shown in Chart 2. The MTX transport was linear for at least 15 min in the parent cell line. Initial uptake studies (15 min) revealed that the resistant cells transported MTX 75% less than did the sensitive cells at MTX concentrations varying up to 25 $\mu$M. The MTX efflux studies revealed similar properties for both sensitive and resistant cell lines (Chart 3).

Kinetic analysis of the initial uptake of MTX at various concentrations (2.5 to 10 $\mu$M) revealed a similar $K_m$ of 6.6 $\mu$M for both cell lines (Chart 4). The $V_{max}$ values, however, are 12.5 and 1.7 nmol/2.5 min/10$^7$ cells for the parent cell line and the MTX-resistant subline, respectively, indicating a nearly 80% decrease in the initial MTX uptake into the resistant cell line.

The initial uptake of 5-MeFH$_4$ was only slightly decreased in the resistant subline ($V_{max}$ 5.26 versus 4.76), while the $K_m$ remained the same for both cell lines.

Effects of DCM, DDMP, and TMQ on the MTX-resistant Cell Line. The comparative inhibitory effects of DCM on the parent and MTX-resistant sublines are shown in Chart 5. MOLT-3/MTX was shown to be equally cross-resistant to DCM. The comparative effects of TMQ on the parent and resistant cell lines are illustrated in Chart 6. In contrast to DCM, the MTX-resistant cells were found to more sensitive to TMQ. Thus, $IC_{50}$ was $7.2 \times 10^{-9}$ M for the parent cell and was $3.0 \times 10^{-8}$ M for the MTX-resistant cell, with a 2.4-fold increase in sensitivity. Similar sensitivity of the MTX-resistant cells to DDMP was observed (Chart 7).
Sensitivities to 5-fluorouracil, 1-β-D-arabinofuranosylcytosine, *E. coli* asparaginase, cisplatin, and daunorubicin did not change in MOLT-3/MTX cells as compared to the parent cell line.

Reversal Effects of LV on Folic Acid Antagonist-Induced Growth Inhibitions. Reversal effects of LV on cell growth inhibition of both cell lines produced by MTX are shown in Chart 8. LV reversed equally the MTX-induced growth inhibition of the parent and resistant cells.

In contrast, the reversal effect of LV on TMQ-induced growth inhibition gave an entirely different picture. Thus, the TMQ-induced growth inhibition of the resistant cells was hardly reversed by equimolar LV, whereas that of the parent cells was easily reversed by LV at any concentrations of TMQ studied (Chart 9). A similar result was obtained when cell growth inhibition of both cells was compared in the presence of equimolar concentrations of DDMP and LV (Chart 10).

DISCUSSION

The acquisition of resistance is a major problem in cancer chemotherapy in humans. Acquired resistance against MTX...
has been related to elevated levels of dihydrofolate reductase (3, 5), structurally altered dihydrofolate reductase (2), or an impairment in the membrane transport of MTX (21, 24). In the present study, although dihydrofolate reductase levels of the resistant cell line MOLT-3/MTX, were slightly elevated (1.4-fold in [3H]dihydrofolate assay) as compared to that of the parent cells, it does not appear to be of sufficient magnitude to be the primary site of resistance. The turnover numbers for MOLT-3 and MOLT-3/MTX, are within the range of those in fresh human tumor dihydrofolate reductase 385 ± 20.6 Based on the data that there was a more than 7-fold decrease in the initial uptake of MTX into the resistant cells, that Km values were similar, and that there was a lack of significant difference in the efflux patterns of the drug between sensitive and resistant cell lines, it is concluded that the development of resistance to MTX is associated with a decreased ability to transport the drug. The morphological differences in the colonies between the parent and resistant cells on the replica plate support the possibility that the membrane characteristics had indeed changed. We observed that the efflux of MTX appeared to be much more rapid than the influx. No complete explanation is available for this observation, but the finding is consistent with that made on murine L1210 leukemia cells (25).

The parent MOLT-3 cells transported 5-MeFH4 at an initial rate more than 2-fold faster than that of MTX, while the Km values for both of these substrates are similar. In L1210 cells, 5-MeFH4 and MTX share a common transport site, and the Km and V max are similar. These differences between mouse and human cells may help to discriminate the cytotoxic differences of MTX seen between the 2 cell lines. As to why this occurs, there are at least 2 possibilities: (a) Although the Km's are similar for 5-MeFH4 and for MTX in human cells, the specificity of binding to the carrier protein may be different, which may account for a less efficient initial uptake of MTX into the human cells and a possible site of alteration for developing resistance to MTX; (b) a possibility exists that there is an additional transport site for 5-MeFH4 uptake that may have a similar Km.

5 B. A. Kamen, J. R. Bertino, M. White, and P. Nylor, unpublished data. Human tumor specimens tested include 3 with Wilms' tumor, 2 with acute lymphoblastic leukemia, and one each with Burkitt's lymphoma and hepatoma.

With regard to the apparent discrepancy between the 4- to 5-fold difference in uptake and the 30-fold difference in growth inhibition assay for the parent and resistant cells, the following observations can be made. (a) Uptake studies were performed over a 15-min period in EBSS, which is used routinely for transport studies of low-molecular nutrients (6), whereas growth inhibition assays were compared after 3 days of incubation in the continuous presence of MTX in the medium. It has been indicated that the intracellular exchangeable MTX levels must be excessive for MTX to inhibit cell growth effectively (9, 26). (b) Although labeled MTX had been purified on the day of the experiment, it cannot be completely ruled out that a part of the radioactivities transported into the resistant cells may in fact be some labeled breakdown products of MTX (14). This question may be answered by dialysis and/or by chromatographic procedures.

Development of MTX-resistant human cell lines by gradient MTX feeding has been reported (21, 24). In the former case, where the parent cell line was derived from the spleen of a patient with spherocytic anemia, cells became resistant due to an elevated dihydrofolate reductase, an impaired membrane transport, or both. The latter case, wherein the cell line was derived from leukemic blasts of a patient with acute lymphoblastic leukemia, was shown to have an impaired membrane transport.

Bertino (1) has suggested the possibility of effective cell kill for MTX-resistant cells by virtue of impaired transport by utilizing folate antagonists that were more efficiently transported by the mutant line. As an example of this phenomenon, Walker carcinoma 256 resistant to MTX was shown to be highly sensitive to triazine (Baker's antifol) (27). In addition, Rosowsky ef al. (24) have indicated that CEM/MTX cells, which were resistant to MTX by impaired transport, showed collateral sensitivity to DDMP as well as di-n-butyl ester of MTX. TMQ (2, 7) and DDMP (18, 20, 23) are lipid-soluble folate antagonists. These antifolates are known to enter cells by a mechanism different from MTX. Our observation that the MTX-resistant cells developed by a gradient MTX feeding were transport related and are more sensitive to TMQ and DDMP than are the parent cells is consistent with these reports.

As a biochemical basis for the collateral sensitivity observed in the resistant cells, Rosowsky et al. have suggested 2 possibilities: (a) the insufficient uptake of reduced folate present in culture medium containing 10% FBS; or (b) some structural alteration of plasma membrane which rendered it more permeable to lipid-soluble agents. In our reversal experiments using LV, the reversal effects of LV against equimolar MTX in the resistant cells, to which much higher MTX and LV were added than in the parent cells, were essentially proportional to those in the parent cells. This would indicate a competitive nature between MTX and LV on the membrane transport where they share a common carrier system in both cells (8, 19) and that the ability to transport reduced folate in the resistant cells was impaired to almost the same degree as was MTX. Our observations indicate, therefore, that the former possibility suggested by Rosowsky et al. is operative as a mechanism of the collateral sensitivity of resistant cells to TMQ and DDMP. The observation of the ability of TMQ and DDMP to effectively kill the MTX-resistant cells with impaired drug transport appears
important in light of the fact that the initial event of the MTX resistance in human cancers is most probably the decreased ability of the drug to penetrate the cell membrane, rather than elevation of dihydrofolate reductase activity (15, 21).

Furthermore, we believe that our observation that TMQ plus LV or DDMP plus LV selectively inhibited the MTX-resistant human tumor cells has important implications. This observation, previously made in the murine lymphoma cell line L5178Y (12), raises a possibility that selective cell kill of transport-resistant neoplasms can be accomplished in humans with little toxicity on host tissues. Thus, the resistant cells with an impaired common reduced folate-MTX transport system will not be protected by LV, whereas host normal cells with an intact reduced folate transport system would be protected effectively from the inhibitory effects of these antifols by using sufficient doses of LV. This thesis can be tested in humans.

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REFERENCES

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