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Natural Resistance to Methotrexate in Human Melanomas

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Human melanomas are naturally resistant to methotrexate (MTX). The mechanism of intrinsic drug resistance has been explored in 3 melanoma cell lines not previously exposed to this agent. All 3 lines exhibited relative MTX resistance with ID_{50} values of greater than 1 μM. Drug uptake studies were performed over an extracellular concentration range of 0.1 to 10 μM MTX. The uptake was linear over the initial 10 min at all concentrations and subsequently reached plateau levels only at the 10 μM concentration. Lineweaver-Burke transformations yielded apparent Km (uptake) values of 1.4 to 5 μM, similar to data obtained from other human cell lines. The level of dihydrofolate reductase (DHFR) in the human melanoma cells ranged between 8.42 to 11.98 pmoles/mg protein. The melanoma DHFR levels are several fold higher than in MTX-sensitive human tumor lines and up to a hundred-fold higher than that measured in human brain tumor cells by our assay. The intrinsic resistance of these melanoma lines has therefore been attributed to elevated intracellular levels of DHFR.

Human melanomas are frequently disseminated at the time of diagnosis. This poses a significant clinical problem since melanomas are resistant to treatment with most chemotherapeutic agents [1]. We have, therefore, chosen human melanomas as a model for the study of natural drug resistance. This report deals with mechanisms of methotrexate (MTX) resistance in these cells.

MTX inhibits the enzyme dihydrofolate reductase and depletes cells of tetrahydrofolate, a cofactor required for the *de novo* synthesis of thymidylic acid and purines [2–4]. The resultant cytotoxicity of MTX, however, can be limited by the ability of malignant cells to develop resistance to the drug. Mechanisms of MTX resistance have been characterized by increased intracellular levels of dihydrofolate reductase or by decreased transport of the drug [5–8]. The resistance of human neoplasms and a variety of cultured cells has been attributed to impaired drug transport and/or increased levels of intracellular dihydrofolate reductase (DHFR) [9–13]. The increase in enzyme level in certain murine cells has been shown to be due to selective multiplication of DHFR genes [14].

Although MTX resistance has been primarily studied in cells selected by prior exposure to the drug, it is also relevant to determine natural modes of resistance in unselected cell populations. We show that three human melanomas are relatively resistant to the effects of MTX *in vitro*. The intracellular uptake of MTX in these lines is comparable to other human tumor cell lines. The mechanism of MTX resistance is attributed to elevated endogenous levels of DHFR.

MATERIALS AND METHODS

Melanoma Growth Studies

The growth characteristics of melanoma cell lines have been previously described [15]. Each line was established from metastatic melanoma and continues to have morphologic characteristics of melanin-producing cells. Furthermore, these lines are tumorigenic in nude mice. All cell lines were maintained for at least 6 mo in McCoy's 5A medium supplemented with 15% fetal calf serum, 100 units of streptomycin per milliliter, and 100 μg penicillin per milliliter. Single cell suspensions were inoculated into Linbro multiwell tissue culture plates (10^6 cells/well) and were allowed to attach for 24 hr prior to exposure. Cells were in log phase growth at the time of drug exposure. Methotrexate (Lederle Laboratories Division, Pearl River, NY) was freshly prepared in medium without serum at a concentration of 0.1 mM. The drug was sterilized by Millipore filtration and serial dilutions were made to give final MTX concentrations of 10^{-4} to 10^{-7} M. Cells were then exposed to various concentrations of the drug for 48 hr. Cells were harvested and counted in a Model Z coulter counter. Results are expressed as the percentage of growth inhibition according to the formula: [(number of cells—number of treated cells)/number of control cells] × 100 after correction for initial densities. Values represent mean ± standard deviation of the mean for 5 to 6 determinations.

MTX Uptake Studies

MTX uptake studies were performed in exponentially growing cells at 72 hr after seeding. The cells were first washed 3 times with culture media without serum at 4°C. The [^{3}H]MTX (Amersham Searle, Arlington Heights, Ill.), specific activity 20 Ci/mmole, was initially diluted 20 fold with unlabeled MTX and subsequently added to the cells at various concentrations in culture media without serum. Incubations were performed at either 37°C or 4°C during a time course up to 40 min. The cells were then washed 10 times with 3 ml of cold phosphate buffered saline. This procedure has been shown to remove extracellular and adsorbed MTX without effecting intracellular (free or enzyme-bound) MTX [16]. The intracellular MTX was measured by dissolving the monolayer in 2 ml of 0.1 M NaCl, 0.01 M Tris-HCl (pH = 7.4), 0.001 M EDTA and 0.5% sodium dodecyl sulfate and counting the recovered radioactivity in a Beckman Model LS-335 liquid scintillation counter after the addition of 13 ml of Aquasol (New England Nuclear, Boston, MA). Aliquots for each determination were assayed for protein using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) (17). All assays were performed in duplicate and repeated twice.

DHFR Assays

DHFR levels were determined on cells in logarithmic growth by measuring the extent of complex formation with [^{3}H]MTX in a ligand-binding assay [18].

RESULTS

Figure 1 shows the growth inhibition of 3 different human melanoma cell lines 48 hr after continuous exposure to various concentrations of MTX. Melanomas NH (Fig 1*A*) and G361 (Fig 1*C*) have similar growth inhibition curves with ID_{50} values of 4×10^{-6} M and 2×10^{-6} M, respectively. HM-1 cells (Fig 1*B*) were relatively more resistant, however, and failed to reach 50% growth inhibition even at 10^{-4} M MTX.

MTX uptake studies for the 3 melanoma lines are shown in

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Abbreviations:
DHFR: dihydrofolate reductase
MTX: methotrexate

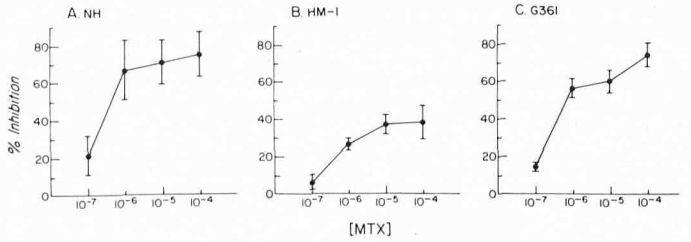

FIG 1. Growth inhibition of human melanoma cell lines at 48 hr after continuous exposure to the various concentrations of MTX.

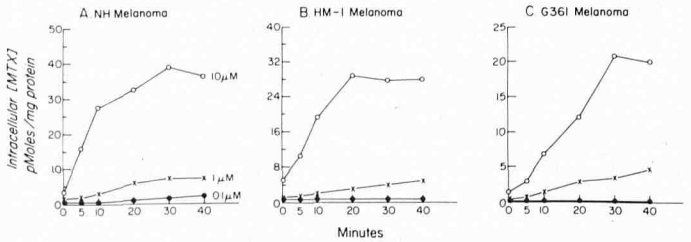

FIG 2. MTX uptake studies for the 3 melanoma cell lines in the presence of extracellular MTX concentrations of 10 μM (○——○), 1 μM (×——×), and 0.1 μM (●——●).

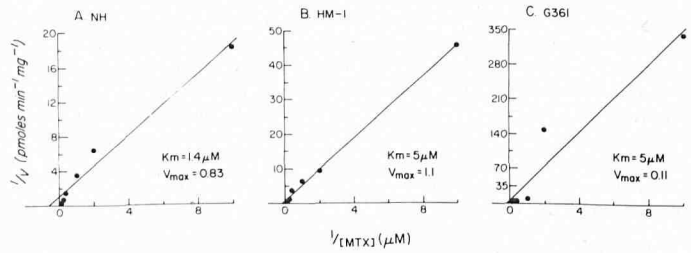

FIG 3. Lineweaver-Burke transformations for the 3 melanoma lines calculated from the initial 10 min of linear MTX uptake for concentrations of 0.1, 0.5, 1.0, 3.0, 5.0 and 10.0 μM.

Fig 2. Constant extracellular MTX concentrations were maintained over a range of 0.1 to 10.0 μM. All determinations were performed at both 37°C and 4°C to differentiate the carrier-mediated/facilitated process from passive diffusion or nonspecific adsorption. Values obtained for uptake at 4°C varied directly with the extracellular MTX concentration. At 1.0 μM extracellular MTX, this constituted approximately 20% of the total uptake [19]. The data in Fig 2 is, therefore, corrected for uptake obtained at 4°C for each concentration shown. MTX uptake is linear through 10 min, following which the rate of accumulation slows at the higher extracellular MTX concentrations and reaches equilibrium. All 3 lines continue to accumulate drug at extracellular concentrations of 0.1 and 1.0 μM over the 40-min time period.

The intracellular MTX appears to achieve a plateau level with an extracellular MTX concentration of 10.0 μM. This suggests that MTX influx and efflux rates are equivalent. However, at the lower extracellular MTX concentrations, the continued accumulation of drug suggests a higher influx than efflux velocity. MTX efflux studies for the 3 melanoma lines were performed following exposure to an extracellular concentration of 10 μM MTX. The efflux was rapid during the initial 5 min and reached plateau levels at 40 min of 14.6, 10.6 and 7.2 pmoles/mg protein for the HM-1, G361 and NH lines, respectively.

The Lineweaver-Burke transformations of MTX uptake for each of the 3 melanoma lines are shown in Fig 3. The apparent Km and Vmax values were calculated during the initial 10 min of linear MTX uptake for 6 different MTX concentrations of 0.1, 0.5, 1.0, 3.0, 5.0 and 10.0 μM. The line plotted in each case was determined using a least squares linear regression analysis. The correlation coefficient for the individual plots is 0.99 for NH, 0.99 for HM-1 and 0.95 for G361.

Dihydrofolate reductase levels in human melanoma cells

Line	DHFR level[a]
NH	11.98
HM-1	8.90
G361	8.42

[a] p moles DHFR/mg protein.

Dihydrofolate reductase levels were also measured in these cells harvested during a period of logarithmic growth. The amount of enzyme activity was normalized to the protein content of the cell extract to give the levels in terms of pmoles DHFR/mg protein. The results listed in the Table for each line represent those obtained for duplicate samples. Assays performed on these cells following several months of continuous passage in culture yielded comparable results.

DISCUSSION

Human melanomas are clinically resistant to MTX even in the high dose protocols [19]. The 3 established melanoma cell lines used in this study were obtained from patients who had not received MTX therapy. These cell lines demonstrate substantial resistance to MTX *in vitro* based upon ID_{50} values similarly obtained for a variety of human and mammalian cell lines [20]. The ID_{50} values of 2×10^{-6} M for the NH and G361 cell lines are 10 to 100-fold higher than values previously reported [20] while the HM-1 line is even more resistant, failing to reach an ID_{50} value at 10^{-4} M MTX.

To delineate the mechanisms of intrinsic resistance to MTX, we have explored both MTX uptake as well as the intracellular levels of DHFR, the putative cytotoxic target of MTX. The MTX resistance in these melanoma lines is not due to a transport deficiency. All 3 lines transport and accumulate at least 20 picomoles of MTX per mg of protein when exposed to extracellular MTX concentrations of 10 μM for a period up to 40 min. This degree of intracellular MTX accumulation is consistent with that previously reported for studies using MTX-sensitive 3T6 cells [16]. Further, more recent studies using the MTX-sensitive human breast carcinoma line, MCF-7, have yielded MTX transport and accumulation data comparable to that obtained with the melanoma cells (data not shown). These intracellular MTX levels reflect only the carrier-mediated or carrier-facilitated uptake in that they have been corrected for any nonspecific uptake by passive diffusion or adsorption as measured at 4°C. The degree of "uptake" at 4°C with extracellular MTX concentrations of 10^{-6} M is consistent with that previously published for human tumor cells [9].

The apparent Km (uptake) values obtained for all 3 melanoma lines (1.4 to 5 μM) are consistent with values previously published for other human tumor cells [9,10]. MTX enters these cells by an active transport process and reaches an equilibrium or steady-state after exposure to extracellular MTX concentrations of 10 mM for 30 min. The steady-state levels indicate that the rates of MTX influx and efflux are equivalent. The efflux process may be limited by binding MTX to intracellular DHFR and by polyglutamation, thus preventing the transport of the drug back to the extracellular medium. The efflux studies indicate rapid MTX loss during the initial 5 min. All 3 lines then reach plateau MTX levels by 40 min and retain between 9 and 15 picomoles MTX/mg of protein intracellularly. These nonexchangeable MTX levels are presumably due to that bound to DHFR.

The resistance of these cells, therefore, appears to be unrelated to the intracellular uptake or accumulation of MTX and suggests a mechanism more directly related to DHFR. Measurements of intracellular DHFR in these human melanomas

have shown that the levels are higher than in a variety of other human cells [18,22]. These levels are also 2 to 3-fold higher than that obtained with human brain tumor cells [18]. Further, there may be a relationship between the DHFR level and degree of MTX resistance. The NH line which had the highest DHFR level was also most resistant to MTX growth inhibition. However, the less than 30% elevation in DHFR level would not fully explain the marked increased resistance of the NH cell line over the other 2 melanoma cell lines. It is known that the K_i values for MTX inhibition of DHFR vary over a wide range from the most sensitive to the more resistant lines [22]. An intrinsic alteration in the K_i for MTX, in addition to the higher levels of intracellular enzyme, may therefore help account for the resistance of these cell lines and the NH cell line in particular.

The high enzyme levels in these lines provide a source for the purification of human DHFR. It should also be feasible to enhance these already high endogenous DHFR levels by continuous exposure to appropriate concentrations of MTX. The melanoma cell lines could therefore serve as a source to purify the mRNA for human DHFR which could lead to the development of probes to examine gene copy number in human tumors sensitive and resistant to MTX. DHFR gene duplication has been identified in sarcoma 180 cells resistant to MTX [14]. Since human melanomas are resistant to most chemotherapeutic agents, these cell lines may also be useful for studying intrinsic resistance to a variety of other drugs.

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REFERENCES

1. Luce JK, McBride C, Frei E III: Melanoma, Cancer Med. Edited by JF Holland, E. Frei III. Lea and Febiger, Philadelphia, 1974, p 1823
2. Chabner BA, Myers EC, Coleman CN, Johns DG: The clinical pharmacology of antineoplastic agents. N Engl J Med 292:1107–1113, 1975
3. Goldman ID: The mechanism of action of methotrexate. I. Interaction with a low affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. Mol Pharmacol 10:257–274, 1974
4. Hyrniuk WM: Purineless death as a link between growth rate and cytotoxicity by methotrexate. Can Res 32:1506–1511, 1972
5. Jackson RC, Niethammer D, Heunnekens FM: Enzyme and transport mechanisms of amethopterin resistance in L1210 mouse leukemia cells. Can Biochem Biophys 1:151–155, 1975
6. Fisher GA: Defective transport of amethopterin as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells. Biochem Pharmacol 11:1233–1234, 1962
7. Harrap KR, Hill BT, Furness ME, Hart LI: Sites of action of amethopterin: Intrinsic and acquired resistance. Ann NY Acad Sci 186:312–324, 1971
8. Fischer GA: Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells. Biochem Pharmacol 7:75–77, 1961
9. Bender RA: Membrane transport of methotrexate in human neoplastic cells. Can Chemother Repts Part 3. 6:73–82, 1975
10. Warren RD, Nichols AP, Bender RA: Membrane transport of methotrexate in human lymphoblastoid cells. Can Res 38:668–671, 1978
11. Niethammer D, Jackson RC: Changes in molecular properties associated with development of resistance against methotrexate in human lymphoblastoid cells. Eur J Can 11:845–854, 1975
12. Chello PL, McQueen CA, DeAngelis LM, Bertino JR: Elevation of dihydrofolate reductase, thymidylate synthetase and thymidine kinase in cultured mammalian cells after exposure to folate antagonists. Can Res 36:2442–2449, 1976
13. Bertino JR, Sawicki WL, Cashmore AR, Cadman EC, Skeel RT: Natural resistance to methotrexate in human acute nonlymphocytic leukemia. Can Treat Repts 61:667–673, 1977
14. Alt FW, Kellems RE, Bertino JR, Schimke RT: Selective multiplication of dihydrofolate reductase genes in methotrexate resistant variants of cultured murine cells. J Biol Chem 253:1357–1370, 1978
15. Wick MM, Byers L, Frei E III: L-dopa: selective toxicity for melanoma cells in vitro. Science 197:468–469, 1977
16. Johnson LF, Fuhrman CL, Abelson HT: Resistance of resting 3T6 mouse fibroblast to methotrexate cytotoxicity. Can Res 38:2408–2412, 1978
17. Bio-Rad Laboratories Technical Bulletin, 1051, April, 1977
18. Abelson HT, Gorka C, Fosberg M, Kornblith P: Identification of dihydrofolate reductase in human central nervous system tumors. Lancet, Jan. 28:184–185, 1978
19. Frei E III. Blum R, Pitman S, Kirkwood J, Henderson I, Skarin A, Mayer R, Bast R, Garnick M, Parker L, Canellos G: High-dose methotrexate with citrovorum factor rescue: rationale spectrum of antitumor activity. Am J Med, 68:370–376, 1980
20. Foley GE, Lazarus H: The response in vitro, of continuous cultures of human lymphoblasts to chemotherapeutic agents. Biochem Pharm 16:659–664, 1967
21. Rosowsky A, Lazarus G, Yuan G, Beltz W, Mangini L, Abelson H, Modest E, Frei E III: Effect of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblast. Biochem Pharmacol, in press
22. Jackson RC, Hart LI, Harrap KR: Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductases. Can Res 36:1991–1997, 1976