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Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture

(active transport/polyglutamation/dihydrofolate reductase/gene amplification/gene expression)

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ABSTRACT **Four methotrexate (MTX)-resistant sublines of a human squamous cell carcinoma (SCC15) were established in culture by progressive dose escalation. The biochemical basis of resistance was studied. The line with the lowest resistance (R1) had a normal dihydrofolate reductase (DHFR) content but showed decreased MTX transport and polyglutamation. Lines of intermediate resistance (R2 and R3) showed an increased DHFR content and DHFR gene copy number and a defect in MTX transport. The line with the greatest resistance (R4) showed increased DHFR content and gene copy number but nearly normal MTX transport. These results demonstrate that multiple mechanisms of MTX resistance occur in human epithelial cells in culture. We also find evidence of alterations in DHFR gene expression. The MTX-resistant cells were either not cross-resistant or only partly cross-resistant to two lipophilic MTX ester derivatives. These compounds are of potential therapeutic interest for the treatment of MTX-resistant tumors.**

Methotrexate (MTX) is among the most active known drugs for clinical cancer therapy (1). For example, in patients with head and neck carcinoma, a 60% response is achieved upon initial treatment (2). However, the response is short and dose escalation is limited by the steepness of the dose–response curve for MTX in man. Much is known about the biochemistry of MTX resistance in murine (3–11) and human (12–19) neoplastic cells in culture, but these studies often involve highly resistant lines with little relevance to the clinic. We chose to study MTX resistance in a cell line (SCC15) derived from a patient with a squamous cell carcinoma of the tongue (20). Here we report that MTX resistance in SCC15 cells arises via several mechanisms, including decreased MTX transport, loss of MTX polyglutamation activity, amplification of the genes for dehydrofolate reductase (DHFR), and possible alteration of *DHFR* gene expression. We also report that lipophilic MTX esters are toxic to SCC15 cells that are resistant to unmodified MTX.

MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and hydrocortisone (0.4 μg/ml). Plating efficiency was 10–20%. In cytotoxicity assays, cells were incubated in folate-free improved modified essential medium (IME medium) containing L-glutamine and antibiotics but no fetal bovine serum.

Cells. A human head and neck squamous cell carcinoma line (SCC15) was obtained from James Rheinwald (Dana–Farber Cancer Institute) (20). To obtain resistant sublines, DME medium containing 20% dialyzed fetal bovine serum was inoculated with cells (2×10^5 per dish), and 24 hr later 50 nM MTX was added. The MTX concentration was increased 1.2- to 2-fold at 1- to 4-week intervals over 13 months. Four resistant sublines were selected: SCC15/R1, 4 months; SCC15/R2, 6 months; SCC15/R3, 10 months; SCC15/R4, 13 months. Doubling times were determined to ensure that the sublines were cytokinetically similar to the parent cell. The stability of MTX resistance was assessed by allowing cells to grow in MTX-free medium for several months and testing them again for MTX sensitivity.

DHFR Content. The amount of enzyme was measured according to Kamen *et al.* (21) by using sonicates pooled from $3–6 \times 10^7$ cells per experiment. Cells were kept in MTX-free medium for several generations prior to the assay.

***DHFR* Gene Assays.** Cellular DNA was extracted with phenol/$CHCl_3$ (22) for Southern blot analysis (23). Probes were prepared from pHD84 plasmid provided by Giussepe Attardi (California Institute of Technology, Pasadena, CA). Hybridization of the human *fes* gene (24) probe to the SCC15 cell DNAs served as a control. To estimate gene copy number, a 20-fold dilution of known amounts of DNA was denatured (0.3 M NaOH, 80°C) and neutralized with 1 M NH_4OAc. The DNA on the filter was adsorbed onto nitrocellulose paper and hybridized with the ^{32}P-rich translated pHD84. Autoradiographs of Southern blots and dot blots (25) were quantitated by densitometry.

Drugs. MTX was the sterile Na salt supplied by the National Cancer Institute. [3′,5′,7-^3H]MTX, hereafter called [^3H]MTX, was from Amersham and was 95% radiochemically pure after chromatography on DEAE-cellulose (3% NH_4HCO_3). The [^3H]MTX in uptake and polyglutamation experiments was purified by HPLC (C_{18}, 3% NH_4HCO_3/0.01 M NH_4OAc, pH 7.9). Solutions were prepared by adding nonradioactive MTX to the desired final concentration and specific activity. Di-*n*-butyl MTX (nBu$_2$MTX) and γ-*t*-butyl MTX (γ-*t*BuMTX) were synthesized as described (26, 27). Stock solutions were made up in 95% ethanol (nBu$_2$MTX) or DME medium (γ-*t*BuMTX) and were sterile-filtered before use.

Cytotoxicity. Cells were tested for sensitivity to MTX, nBu$_2$MTX, and γ-*t*BuMTX in a colony-counting assay in DME medium containing 20% dialyzed fetal bovine serum (1000–2000 cells inoculated per plate). Drugs were added at 24 hr; after *ca.* 2 weeks, the colonies were fixed, stained (methylene blue), and counted.

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Abbreviations: MTX, methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamate); DHFR, dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3); nBu$_2$MTX, di-*n*-butyl methotrexate; γ-*t*BuMTX, γ-*t*-butyl methotrexate.

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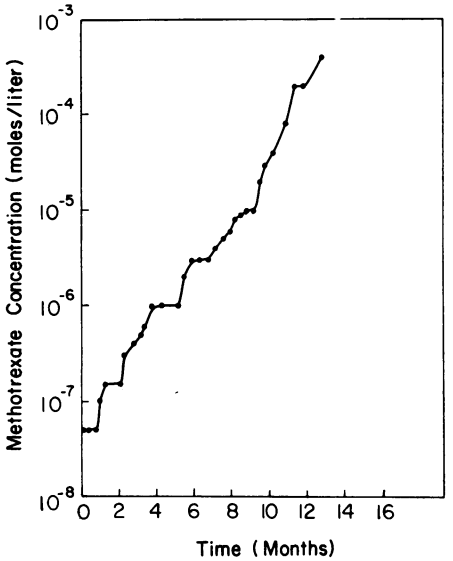

FIG. 1. Time course for the development of MTX resistance in human squamous cell carcinoma of the head and neck (SCC15) in culture.

MTX Transport. Seven 100-mm plates of 0.8–1 × 10^6 cells in 10 ml of DME medium with 20% dialyzed fetal bovine serum were used in each experiment. After 2 days in MTX-free medium, the cells were washed with IME medium containing 2 mM L-glutamine and conditioned by incubation at 37°C for 1 hr. Uptake was initiated by replacing the medium with 10 ml of 2 μM [^{3}H]MTX in IME medium. At 2-min intervals the medium was aspirated, and the cells were washed with ice-cold phosphate-buffered saline and digested in 2.5 ml of 1 M NaOH at room temperature overnight for counting (Dimiscint, National Diagnostics, Somerville, NJ). Proteins were measured in the digest (28), and the [^{3}H]MTX content in pmol/mg of protein was calculated. Since we sought to determine total uptake including passive diffusion, measurements at 4°C were not performed. However, a correction for background and cell-surface binding was made at each time point so that uptake plots intersected the origin.

Polyglutamate Synthesis. The method recently described (29) was used.

RESULTS

Development of Resistance. The MTX concentration tolerated by SCC15 cells exposed to MTX increased progressively from 50 nM to 0.2 mM over a 1-year period (Fig. 1). Separate cultures were established at 4, 6, 10, and 13 months, at which time the MTX concentrations were 15 (R1), 90 (R2), 433 (R3), and 4000 (R4) times the starting dose. Fig. 2 shows the dose–response curve for each line. As indicated in Table 1, the IC_{50} increase ranged from 17-fold (R1) to 9000-fold (R4). The doubling time for all of the lines was 1.5–1.7 days. R1 cells remained about 17-fold resistant for 4 weeks in the absence of MTX but were only 3-fold resistant by 8 weeks. R4 cells retained a high level of resistance for at least 4 months in the absence of selective pressure.

MTX Uptake. The uptake of [^{3}H]MTX in folate-free medium is presented for all five SCC15 cell lines in Fig. 3. The initial uptake velocities are listed in Table 1. The data show that the R1, R2, and R3 cells were markedly defective in MTX uptake. The unidirectional influx rate was 5- to 10-fold lower than that of the parental cell. Only a small decrease in uptake velocity was observed in R4 cells.

Formation of MTX Polyglutamates. The extent of MTX polyglutamation varied markedly between the parental and R1 cells (Table 2). After 24 hr of treatment with 2 μM MTX, the total intracellular drug concentration was 20–25 pmol/mg of protein for both cell lines. In the presence of 20 μM MTX, this concentration increased to about 275 pmol/mg of protein. Whereas in the parental cells 70% of the labeled MTX was found to be polyglutamated, essentially all the MTX in R1 cells remained unchanged. When extracellular MTX was increased to 20 μM, only 30% of the MTX in the parental cells was present as polyglutamates, suggesting saturation of the polyglutamate synthetase system. In contrast, the MTX in R1 cells was only 6% polyglutamated. Marked differences also were seen when the distribution of polyglutamates was compared. Whereas all the polyglutamates from MTX(G_1) to MTX(G_5) were observed in the parental SCC15 cells, the only detectable species in R1 cells at 2 μM was MTX(G_1). When MTX was increased to 20 μM, small amounts of MTX(G_2) – MTX(G_4) were formed, but MTX(G_5) remained undetectable.

When R3 cells were incubated with 2 μM MTX for 24 hr, the total uptake was increased 2-fold relative to the parental and R1 cells, but with 20 μM extracellular drug, the uptake in all three cells was essentially the same. No detectable polyglutamation was seen at 2 μM MTX, and only MTX(G_1) (<2%) was observed at 20 μM MTX.

DHFR Content. The ability of DHFR in cell lysates to bind [^{3}H]MTX was measured (Table 1). The binding activity of R1 cells was somewhat less than that of the parent line. The binding activity of the other cells was elevated 4.3- to 276-fold relative to the parent line.

***DHFR* Gene Amplification.** Increased DHFR levels have been ascribed to increased gene copies of the *DHFR* locus (7, 17, 18, 30, 31). To measure the number of gene copies homologous to *DHFR*, total cellular DNA was cleaved with restriction enzymes, and DNA fragment homologous to the *DHFR* gene were detected by Southern blot analysis with a

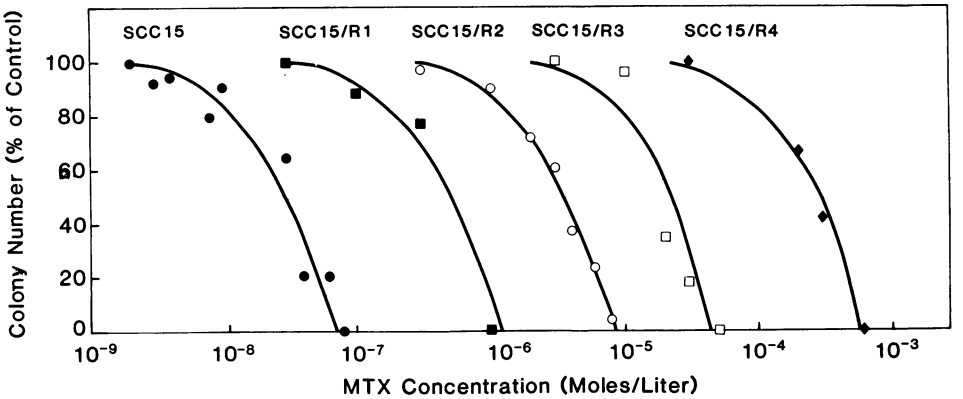

FIG. 2. Dose–response curves for MTX against MTX-sensitive and MTX-resistant SCC15 cell lines in culture.

Table 1. MTX uptake, DHFR content, and sensitivity to lipid-soluble antifolates in MTX-sensitive and MTX-resistant human squamous cell carcinoma of the head and neck (SCC15) in culture

Cell line	[^{3}H]MTX uptake,* pmol/min per mg of protein	DHFR content		Sensitivity to antifolates,† IC_{50}, μM		
		[^{3}H]MTX bound, pmol per 10^8 cells	Relative no. of *DHFR* copies	MTX	nBu$_2$MTX	γ-tBuMTX
SCC15	0.39 ± 0.02	5.4 (1)	1.0	0.030 (1)	0.40 (1.0)	0.60 (1.0)
SCC15/R1	0.06 ± 0.03	5.0 (0.91)	1.0	0.50 (17)	0.64 (1.6)	1.3 (2.2)
SCC15/R2	0.03 ± 0.04	23 (4.3)	2.7	3.6 (120)	3.7 (9.2)	6.7 (11)
SCC15/R3	0.07 ± 0.06	395 (73)	4.4	22 (730)	3.4 (8.5)	40 (67)
SCC15/R4	0.24 ± 0.05	1490 (276)	10	270 (9000)	22 (55)	54 (90)

*Mean ± SD (uptakes for R1, R2, and R3 cells are not statistically distinguishable from zero).
†In the nBu$_2$MTX assays, ethanol was present at the following final percentage at the IC_{50}: SCC15, 0.01; SCC15/R1, 0.0?; SCC15/R2, 0.1; SCC15/R3, 0.1; SCC15/R4, 0.2. In control experiments these percentages of ethanol did not affect growth. Values in parentheses are normalized for each compound relative to the parent SCC15 cell. IC_{50} values are means of at least two separate experiments.

^{32}P-labeled plasmid probe containing a fragment of the human *DHFR* gene (Fig. 4). At least nine discrete DNA fragments homologous to *DHFR* were observed in *Eco*RI-digested DNA. The increase in total number of sequences that hybridized to the DHFR probe, determined from dot blots (data not shown) and from measurement of radioactivity in each fragment in the Southern blot, is summarized in Table 1. The number of gene copies in R1 cells was the same as that in the parental line. Amplification of the *DHFR* homologous sequences was evident in the R2, R3, and R4 cells. The number of gene copies relative to the parental SCC15 cells was elevated 2.7-fold in R2 cells, 4.4-fold in R3 cells, and 10-fold in R4 cells. Two of the DNA fragments in the Southern blot analysis (Fig. 4) did not show an increase in gene copy number in the R2, R3, and R4 lines. We suspect that these fragments may be derived from a pseudogene (32, 33).

Cytotoxicity. The level of resistance of the R1–R4 cells to the lipophilic MTX esters nBu$_2$MTX and γ-tBuMTX was examined (Table 1). The resistance of the cells to the esters was much less than the resistance of the cells to MTX. For example, the IC_{50} of MTX in R1 cells was elevated 17-fold relative to the parent line, whereas the IC_{50} of nBu$_2$MTX was elevated only 1.6-fold. The lipophilic esters also retained significant activity against the more resistant lines—e.g., the R4 cells were 9000-fold resistant to MTX but only 55-fold resistant to nBu$_2$MTX.

DISCUSSION

The ability to produce resistant head and neck carcinoma cell lines *in vitro* by MTX dose escalation provides a laboratory model for the study of biochemical changes associated with MTX resistance. This study was designed, in part, to address the clinically relevant problem of MTX resistance. The dose–response curve for MTX in humans is such that a 5- to 10-fold increase in cellular resistance is manifest as a clinically refractory tumor. Experimental studies using cells that are highly resistant to MTX are of biochemical interest but do not necessarily yield information relevant to the clinic. Therefore, we chose to examine cells with low (R1), intermediate (R2, R3), and high (R4) levels of resistance.

A variety of biochemical alterations contribute to the development of MTX resistance in SCC15 cells. The R1 subline (low resistance) was deficient in both MTX transport and MTX polyglutamation. No increase in the amount of DHFR was detected in the R1 cells. The uptake velocity of

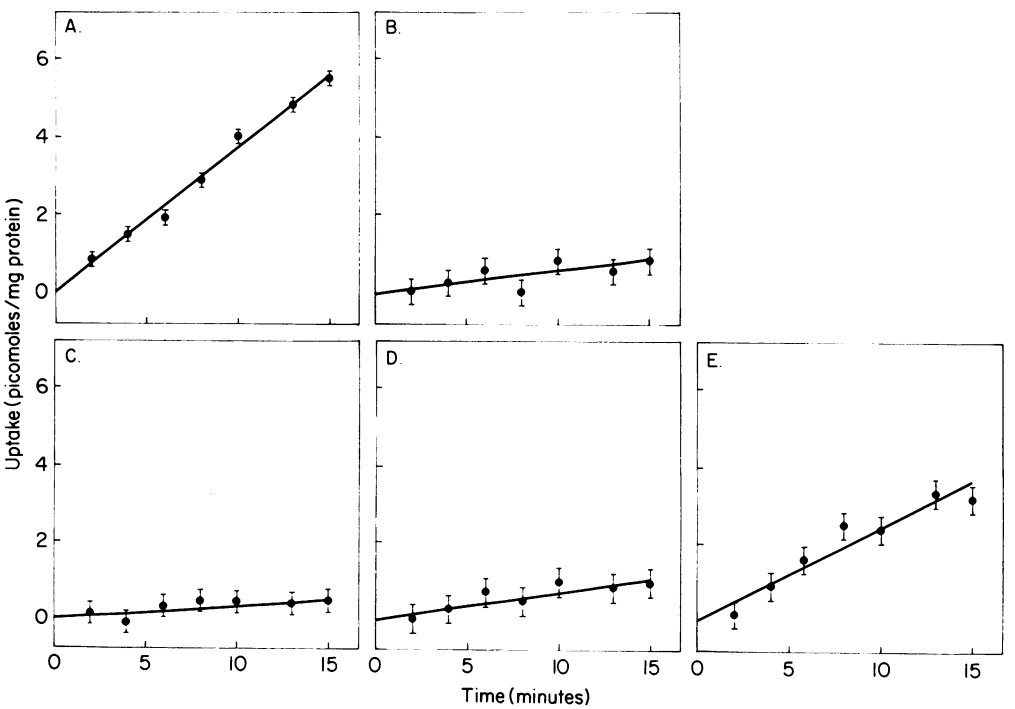

FIG. 3. Uptake of [^{3}H]MTX in MTX-sensitive and MTX-resistant SCC15 cell lines. (*A*) SCC15 cells. (*B*) SCC15/R1 cells. (*C*) SCC15/R2 cells. (*D*) SCC15/R3 cells. (*E*) SCC15/R4 cells. The extracellular [^{3}H]MTX concentration in each experiment was 2 μM. Vertical bars represent ±SD.

Table 2. Polyglutamation of MTX in human squamous cell carcinoma (SCC15) cells in culture

Cell line	Extracellular MTX, μM	Total 24-hr uptake, pmol/mg of protein	Polyglutamates,* % of total at 24 hr					
			$MTX(G_0)$	$MTX(G_1)$	$MTX(G_2)$	$MTX(G_3)$	$MTX(G_4)$	$MTX(G_5)$
SCC15	2	20	29.7	9.1	17.7	20.7	21.4	1.4
	20	275	67.9	5.2	9.7	9.0	8.3	—
SCC15/R1	2	25	98.7	1.1	—	—	—	—
	20	272	93.7	1.2	3.0	1.4	0.7	—
SCC15/R3	2	53	100	—	—	—	—	—
	20	263	98.3	1.7	—	—	—	—

*The terms $MTX(G_n)$ refer to the number of extra glutamates attached to the γ-carboxyl group in the glutamate residue of MTX—e.g., $MTX(G_1)$ is 4-amino-4-deoxy-N^{10}-methylpteroyl-γ-L-glutamyl-L-glutamate; therefore $MTX(G_0)$ represents no polyglutamation of MTX.

MTX was slower in R1 cells than in the parental line. However, by 24 hr the total intracellular concentration of MTX in the two cells was the same.

A defect in polyglutamation was also detected in the R1 cells. Since polyglutamation was measured at 24 hr and since low polyglutamation was seen even with 20 μM MTX, we conclude that non-polyglutamation was not due to decreased transport. MTX resistance in R1 cells probably reflects a decrease in both processes. The low level of polyglutamation may be due to a decreased level of polyglutamate synthetase itself or to a defect in regulation of the enzymatic activity.

MTX resistance in R2 and R3 cells was associated with overproduction of DHFR and impaired MTX transport. These cells produced 4-fold and 73-fold more DHFR than the parent line. Polyglutamation also may be impaired. However low polyglutamation in these cells could be due to the high level of DHFR, which is known to have a much higher affinity for MTX than does the polyglutamate synthetase (34, 35).

The resistance of R4 cells can be explained only partly by the increased DHFR content. DHFR is elevated 276-fold relative to the parent line, whereas the IC_{50} is increased 9000-fold. We suspect that this very high resistance may reflect another, as yet unidentified, biochemical alteration. One possibility is that the DHFR in the highly resistant cells has a low MTX affinity (5, 10, 36).

The ratio of DHFR enzyme to *DHFR* gene copies is anomalous in R3 and R4 cells. The DHFR content was comparable to the gene number in the parental cells and the R1 and R2 cells, but in the R3 and R4 cells, the difference in gene copy number relative to the parent line was much smaller than the difference in DHFR enzyme. These observations suggest that the rate of expression of the stable gene product in the more resistant cells is 20–30 times that of the normal *DHFR* gene. A similar over-expression phenomenon for DHFR has been noted in human epithelial carcinoma (KB) cells *in vitro* (18). Increased enzyme production per gene has not been observed in S180 murine sarcoma (30) and may be a peculiarity of human cells.

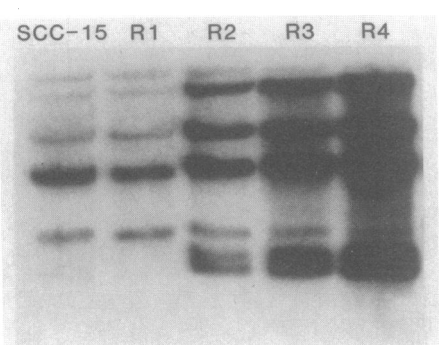

FIG. 4. *Eco*RI-digested DNA from MTX-sensitive SCC15 cells and MTX-resistant sublines (R1–R4) hybridized to the DHFR probe pHD84.

Our finding that MTX-resistant SCC15 cells are either not cross-resistant or only partly cross-resistant to nBu_2MTX (26, 37, 38) and γ-*t*BuMTX (27) is of potential therapeutic interest. We believe these compounds may bypass the normal MTX active transport pathway and penetrate cells by passive diffusion. Cells resistant to MTX as a consequence of defective transport should retain at least partial sensitivity to the esters, as was noted in the R1 line. The 100- to 200-fold difference in IC_{50} between MTX and the MTX esters in R4 cells cannot be explained solely on the basis of transport. We suspect that the intracellular nBu_2MTX and γ-*t*BuMTX concentration, unlike that of MTX, is not limited by active transport and that these lipophilic molecules can accumulate to high levels in the cells—i.e., levels equivalent to the amount of DHFR. Thus, a lipophilic MTX derivative such as γ-*t*BuMTX may be useful in overcoming MTX resistance.

We are examining other head and neck carcinoma lines, including clonally derived cells, to trace the biochemical evolution of MTX resistance in this solid tumor model and to evaluate the utility of lipophilic esters of classical antifolates in overcoming or preventing transport-based resistance.

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