Inherent Resistance of Human Squamous Carcinoma Cell Lines to Methotrexate as a Result of Decreased Polyglutamylation of This Drug

Giuseppe Pizzorno, Yu-Ming Chang, John J. McGuire, and Joseph R. Bertino

ABSTRACT

Three human squamous carcinoma cell lines (FaDu, A253, and SQCC/Y) were tested for sensitivity to methotrexate (MTX) and trimetrexate, a second generation folate antagonist in clinical trials. Two of the three cell lines (A253 and SQCC/Y) showed inherent resistance to methotrexate, when cytotoxicity was evaluated after short term exposure (4 and 24 h). In contrast, all three cell lines were markedly sensitive to trimetrexate, an antifolate which is taken up by cells by a different transport system than is methotrexate and which is not polyglutamylated. The basis for the natural resistance to methotrexate shown by two of the three cell lines was examined. Levels of dihydrofolate reductase activity, inhibition of this enzyme by methotrexate, and influx of MTX did not differ significantly between the three cell lines; however, resistance was correlated to the amounts of polyglutamates of methotrexate synthesized by the three cell lines. After a 24-h incubation with 10 \( \mu \)M MTX, the A253 cell line was able to form only 35.0 pmol/10^7 cells of polyglutamates, compared to 250 pmol/10^7 cells synthesized by the FaDu cell line, while the SQCC/Y cell line, intermediate in sensitivity to methotrexate, was able to form 145 pmol/10^7 cells of MTX polyglutamates. The A253 cell line contained less polyglutamate synthetase activity compared to the FaDu and SQCC/Y cell lines. However, it is not clear if this difference is sufficient to explain the marked differences in polyglutamates of methotrexate found between the cell lines. We conclude that decreased polyglutylation of methotrexate in some human squamous cell carcinomas may be the major contributing factor in inherent resistance to high dose pulse administration of this drug.

INTRODUCTION

The treatment of squamous cell carcinoma of the head and neck remains a difficult problem for the oncologist. Surgery and radiotherapy are the main methods of treatment especially for the early stages of head and neck cancer, where cures may be obtained. However, in patients with large primary lesions and lymph node involvement, curability is low. Since the 1960s, chemotherapy has been evaluated in an attempt to improve the treatment of patients with advanced or recurrent cancer of the head and neck. Active drugs for the treatment of head and neck carcinoma include methotrexate, cis-platinum, bleomycin, and 5-fluorouracil. The response rate with these drugs as single agents is between 15 and 35% (1). Combination chemotherapy regimens appear to increase the percentage of remissions achieved but have not yet led to improved survival (2). MTX, the first anticancer drug used in this disease, is an inhibitor of dihydrofolate reductase and thus results in impaired synthesis of thymidylate, purines, methionine, and serine. MTX, like the physiological folates, is converted to poly-\( \gamma \)-glutaryl derivatives in both normal and malignant cells (3-17). The polyglutamate derivatives not only are retained by the cells after disappearance of extracellular drug, which may result in prolonged cytotoxicity (18-23), but also have a direct inhibitory effect on other enzymes in addition to dihydrofolate reductase, i.e., thymidylate synthase and glycaminide ribonucleotide and aminoimidazolecarboxamidase transformylases (24-26).

In this paper, mechanisms involved in the inherent or 'natural' resistance to MTX of squamous cell carcinomas of the head and neck are reported. Three squamous cell lines, derived from untreated patients, were studied: A253 from an epidermoid carcinoma (27), FaDu from a hypopharyngeal carcinoma (28), and SQCC/Y, from a squamous carcinoma of the buccal mucosa (29).

MATERIALS AND METHODS

Chemicals. MTX was obtained from the National Cancer Institute (Bethesda, MD) and dissolved in NaOH and the pH was adjusted to 7.0. TMQ was obtained from Warner-Lambert/Parke-Davis (Ann Arbor, MI) and dissolved in sterile water. FMTX was a gift from Dr. J. K. Coward, University in Michigan (Ann Arbor, MI). Chemically synthesized MTX polyglutamates containing one to five additional glutamate moieties were obtained from Dr. N. Lomax, National Cancer Institute, and from American Radiolabeled Chemical (St. Louis, MO). Dihydrofolic acid was synthesized by the method of Blakley (30). [5-\(^{3}H\)]-2'-deoxyuridine and [3\(^{5}\)S,\(^{32}\)P]methotrexate were purchased from Moravek Biochemicals (Brea, CA), [\(^{3}H\)]MTX was purified using reverse phase high performance liquid chromatography as previously described (6). All other chemicals were of the highest purity available.

Medium, serum, and antibiotics for tissue culture were purchased from Grand Island Biological Co. (Grand Island, NY). Plasticware was purchased from Corning Glass Works (Corning, NY), Costar (Cambridge, MA), and Nunc (Roskilde, Denmark).

Cell Lines. The squamous cell carcinoma lines (A253, FaDu, and SQCC/Y) were obtained from Dr. M. Reiss (Yale University, New Haven, CT). Cells were tested for Mycoplasma contamination and found to be negative during the course of these experiments. Cultures were maintained, in 25-cm\(^2\) plastic flasks, as monolayers in RPMI 1640 medium that was supplemented with 10% filtered FBS, penicillin (100 IU/ml), and streptomycin (100 \( \mu \)g/ml), at 37°C in a 5% CO\(_2\) atmosphere, and were subcultured once every 4 days after 15-min incubations with 2.5% trypsin and 1% EDTA in 0.9% NaCl solution. Under these conditions the doubling times were 31, 25, and 27 h, respectively, for A253, FaDu and SQCC/Y, (hereafter called SQ).

Growth inhibition Studies. Inhibitory effects on growth of squamous cell lines were determined by measuring cell counts after variable times of exposure to MTX or TMQ (4, 24, 48, or 72 h) and regrowth in drug-free medium for a total of 72 h, except for the 72-h exposure, in which case cells were enumerated immediately after the exposure.

Monolayers of cells in 25-cm\(^2\) sterile flasks were exposed to various concentrations of MTX or TMQ (1.5 \( \times \) 10\(^5\) cells/flask). After 72 h, the medium was removed and the cell layer was washed twice with PBS (140 mm NaCl, 3 mm KCl, 8 mm Na\(^2\)HPO\(_4\), and 1.5 mm KH\(_2\)PO\(_4\)). The monolayer was covered with 1 ml of trypsin-EDTA solution and incubated for 15 min, and then 9 ml of 10% FBS-supplemented RPMI

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The abbreviations used are: MTX, methotrexate; TMQ, trimetrexate; FMTX, \( \gamma \)-fluoromethotrexate; FBS, fetal bovine serum; ED\(_{10}\), the concentration required to decrease the cell count to 50% of untreated control; IC\(_{50}\), the concentration of drug required to inhibit enzyme activity to 50% of untreated control; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate; PBS, phosphate-buffered saline; SQ, SQCC/Y.
medium was added to stop the trypsin reaction. Appropriate dilutions of the cell suspension were counted using a model ZB Coulter counter (Coulter Electronics, Hialeah, FL). For "pulse" treatment, after 4, 24, or 48 h of exposure to different concentrations of drug, the monolayer was washed twice with PBS and allowed to regrow in drug-free medium until 72 h total time. The procedure was then carried out as for the 72-h exposure.

Preparation of Cell Extracts. Exponentially growing cells in 600-cm² tissue culture plates were washed twice with 30 ml of ice-cold PBS and harvested by scraping the cells with a rubber blade into 2 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl, 10% glycerol, and 2 mM dithiothreitol. Crude extracts were obtained by freeze-thawing the cells three times, followed by centrifugation at 16,000 × g for 30 min. A similar procedure was followed for FPGS assays, except that the harvesting buffer was 0.1 M Tris-HCl, pH 8.85, containing 100 mM 2-mercaptopethanol.

Enzyme Assays. DHFR activity was determined spectrophotometrically as described (31). The decrease in absorbance at 340 nm, which occurs when NADPH and dihydrofolate are converted to NADP and tetrahydrofolate, respectively, at 37°C, was measured using a Gilford model 2000 recorder (Gilford Instruments, Oberlin, OH) attached to a Beckman DU spectrophotometer (Beckman Instruments, Fullerton, CA).

IC values for MTX were obtained by inhibition assay, in which the drug was added at various concentrations to the assay mixture which contained enzyme (activity, ΔA 0.040/min at 37°C). Protein concentrations were determined by the semimicro biuret method.

Uptake of MTX. Cells (5.6 × 10⁵ cells/flask) were cultured 18–24 h before counting out the assay in 25-cm² flasks. Uptake was initiated by replacing the medium with 3 ml of 1 μM [3H]MTX (600 cpm/pmol) in RPMI 1640 medium with 10% filtered FBS. At 1-, 2-, 3-, 4-, 5-, 15-, 30-, and 60-min intervals, the medium was aspirated from individual flasks and the adherent cells were washed 3 times with ice-cold PBS and incubated for 1 h in 2 ml of 1 M NaOH. Then 1 ml of NaOH solution was neutralized with 1 ml of 1 M HCl and the radioactivity was quantitated after adding 15 ml of scintillation fluid (8).

Thymidylate Synthase Activity in Situ. The activity of this enzyme in intact cells was measured using [5-3H]-2'-deoxyuridine (32). After cellular uptake of [5-3H]-2'-deoxyuridine and conversion to [5-3H]-dUMP, tritium is released as 3H₂O into the medium when this compound is methylated by thymidylate synthase.

The cells were cultured in 6-well plates, 24 h before carrying out the assay. To 2–3 × 10⁵ cells/well in complete medium, [5-3H]-2'-deoxyuridine was added to a final concentration of 1 μCi/ml and 100-μl samples were taken at 15, 30, 45, 60, 75, and 90 min. These were added to 200 μl of a 15% activated charcoal suspension in 4% trichloroacetic acid, mixed, and centrifuged for 5 min at 16,000 × g. One hundred μl of supernatant were added to 5 ml of scintillation fluid and radioactivity was quantitated using a scintillation counter.

A modification of the method of Rodenhuis et al. (33) was used for the detection and classification of MTX resistance. The cells were cultured in 6-well plates, 24 h before carrying out the assay, to a density of 2–4 × 10⁵ cells/ml in complete medium. Five 1-ml aliquots were incubated for 3 h in the presence of, respectively, no drug, 0.1 μM MTX, 1 μM MTX, 1 μM TMQ, 1 μM TMQ, and 5 μM FMTX. [5-3H]-2'-Deoxyuridine was added to a final concentration of 1 μCi/ml and 100-μl samples were taken at 0, 15, 30, and 45 min. These were added to 200 μl of a 15% activated charcoal suspension in 4% trichloroacetic acid, mixed, and centrifuged for 5 min at 16,000 × g. One hundred μl of supernatant were added to 5 ml of scintillation fluid and radioactivity was quantitated using a scintillation counter.

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RESULTS

Drug Sensitivity Studies. The cytotoxicity of MTX or TMQ on the three squamous cell lines of the head and neck was evaluated using different times of exposure to the drug, 4, 24, 48, and 72 h (Fig. 1).

FaDu was the most sensitive cell line to MTX, showing an ED₅₀ of 3 μM after 4 h of incubation; with increasing duration of exposure, the ED₅₀ decreased to 20 nm (72 h). As compared to the FaDu cell line, A253 is about 30-fold more resistant to MTX for the shortest exposure, 4 h, to MTX; relative resistance decreased to 15-fold for a 24-h exposure, with a further decrease to 2-fold for the 72-h exposure. The ED₅₀ for the 4-h exposure to MTX in SQ, was about 50 μM and was 0.5 μM for the 24-h exposure; for the 48 and 72 h following exposure, the pattern of SQ toxicity was equivalent to that of FaDu.

The effect of TMQ on the A253 and the FaDu cell lines was approximately the same; the ED₅₀ values at 24 h were 0.1 and 0.02 μM, respectively, after a 72-h continuous exposure. The cytotoxicity in the SQ cell line was significantly different; the ED₅₀ values were 0.46 and 0.07 μM at 24 and 72 h, respectively.

Detection and Classification of MTX Resistance. The mechanism of the relative inherent resistance of A253 and SQ to MTX was investigated initially by studying the effects of MTX, TMQ, and FMITX on in situ thymidylate synthase activity (Table 1). The A253 cell line showed moderate sensitivity to all three antifolates after 3 h in the presence of drug. After 4 h of...
expression as percentage of the slope (3H release versus incubation time) of untreated control cells.

medium, suspended in drug-free medium, and incubated for another 6 h. The tritium release assay was carried out as described for the 3-h incubation. The results are in "Materials and Methods." For the efflux, cells incubated in the presence of no drug, 1 μM MTX, 1 μM TMQ, and 5 μM FMTX, washed 3 times with complete medium, thymidylate synthase activity in this cell line was also inhibited by the concentrations used for the three drugs, after 3 h of incubation. After resuspension in drug-free medium, thymidylate synthase activity in this cell line was still inhibited by TMQ and MTX but not by FMTX. This latter compound was used as a positive control, since it was slightly lower. For convenience, aminopterin was used as the pteroyl substrate in these studies, since its substrate activity with human FPGS is much higher than that of MTX (35). To rule out differences in substrate specificity as the reason for the similar specific activities, the substrate activity of MTX was also determined. The substrate activity of MTX relative to aminopterin was about 0.3 in each case (Table 2). Experiments also determined. The substrate activity of MTX relative to aminopterin was about 0.3 in each case (Table 2). Experiments in which extracts were mixed with partially purified CCRF-CEM cell FPGS (35) gave additive activities (data not shown), indicating the absence of both endogenous activators and inhibitors in the extracts.

MTX Free and Bound to DHFR at 30 Min. The possibility that the decreased level of polyglutamates found in the A253 and SQi, as compared to FaDu, cells was due to poor relative uptake, with related lack of free intracellular MTX necessary for polyglutamate formation, or increased intracellular binding led to a study of a 30-min uptake of 1 μM MTX. The amount of free drug, drug bound to DHFR, and MTX polyglutamates were analyzed. At least 40% of the drug was presented as free MTX in all three cell lines (Table 4), with 65% in the FaDu line. At this time point, MTX diglutamate was the only formsynthesized in all lines, about 1 pmol/10^7 cells, even in the cell line FaDu was also inhibited by the concentrations used for the three drugs, after 3 h of incubation. After resuspension in drug-free medium, thymidylate synthase activity in this cell line was essentially the same, 521 and 556 cpm/0.1 ml medium/min, respectively. The level of this enzyme activity in exposure to these drugs, then resuspension of the cells for 6 h in drug-free medium, thymidylate synthase activity was still inhibited by TMQ, but there was complete recovery after MTX and FMTX treatments. In situ thymidylate synthase activity in the cell line FaDu was also inhibited by the concentrations used for the three drugs, after 4 h of incubation. After resuspension in drug-free medium, thymidylate synthase activity in this cell line was still inhibited by TMQ and MTX but not by FMTX. This latter compound was used as a positive control, since it has the same mechanism of action as MTX, namely DHFR inhibition, but it is poorly polyglutamylated (36). SQi was the cell line least sensitive to antifolates, and after efflux there was complete recovery from MTX and FMTX inhibition but not from TMQ inhibition. These results were consistent with impaired polyglutamylation as the cause for natural resistance of the A253 and the SQi lines to short term exposure to MTX. In order to prove further that this was the cause of resistance, the following studies were performed.

Thymidylate Synthase Activity. Thymidylate synthase activity in cell extracts was measured using the release of H_2O after incubation with [5-^3H]deoxyuridine. The activity for A253 and SQi was essentially the same, 521 and 556 cpm/0.1 ml medium/min, respectively. The level of this enzyme activity in

FaDu cells was only one fifth the activity of the other two cell lines (Table 2).

Uptake of [^3H]MTX. Uptake studies using 1 μM [^3H]MTX are presented in Fig. 2. The initial velocities of uptake were 0.84, 1.08, and 0.98 pmol/min/10^7 cells, respectively, for A253, FaDu, and SQi (Fig. 2A). Different behavior was shown in the long term uptake study (Fig. 2B). Uptake of [^3H]MTX in A253 and SQi slowed to a level of 20 and 29 pmol/10^7 cells, respectively, after 30–60 min. FaDu, in the same period of time, showed a higher rate of uptake, about 4-fold greater.

MTX Polyglutamate Formation in Whole Cells. After 24 h of incubation with 10 μM MTX, the amount of MTX polyglutamates formed varied markedly among the three cell lines (Table 3). A253 showed a total intracellular MTX concentration of 86 pmol/10^7 cells and only 15.6 pmol, 18% of the total amount, were MTX polyglutamates with more than 3 glutamates. FaDu had an intracellular drug concentration of 319.7 pmol/10^7 cells and 80.3 pmol of the high-MTX polyglutamates. SQi showed a lower intracellular drug concentration compared to FaDu, 209.6 pmol/10^7 cells, but 75 pmol (35.8% of the total) were MTX with 4–6 glutamate moieties. A253 showed, for the 4-h exposure to MTX, a low polyglutamate formation when compared to the amount accumulated in FaDu cells. Also, the SQi cell line had a lower amount of polyglutamates but in this case the difference from the FaDu cell line was less dramatic.

Table 1 Detection and classification of MTX resistance in squamous carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incubation for 3 h</th>
<th>Efflux 6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX, 0.1 μM</td>
<td>MTX, 1.0 μM</td>
</tr>
<tr>
<td>A253</td>
<td>93.5 ± 3.5 μM</td>
<td>26.3 ± 2.3</td>
</tr>
<tr>
<td>FaDu</td>
<td>87.3 ± 4.5 μM</td>
<td>11.5 ± 1.3</td>
</tr>
<tr>
<td>SQi</td>
<td>99.2 ± 2.5 μM</td>
<td>32.9 ± 3.4</td>
</tr>
</tbody>
</table>

* Mean ± SD from three experiments.
POLYGLUTAMYLAION IN SQUAMOUS CELL LINES

Table 2 Analysis of folate-dependent enzymes in squamous cell carcinoma lines

<table>
<thead>
<tr>
<th>DHFR</th>
<th>Thymidylate synthase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Specific activity (μmol/h/mg)</td>
</tr>
<tr>
<td>A253</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>FaDu</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td>SQ₁</td>
<td>0.23 ± 0.05</td>
</tr>
</tbody>
</table>

Table 3 Intracellular concentration of MTX and MTX polyglutamates in three squamous cell carcinoma cell lines following 4- or 24-h exposure to 10 μM extracellular [³H]MTX

<table>
<thead>
<tr>
<th>Cell line</th>
<th>4-NH₂-10-CH₃-PteGlu₃ (pmol/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A253</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>FaDu</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>SQ₁</td>
<td>0.3 ± 0.3</td>
</tr>
</tbody>
</table>

Table 4 MTX bound and free and its metabolism after a 30-min incubation at 1 μM

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total MTX (pmol/10⁷ cells)</th>
<th>Bound MTX (pmol/10⁷ cells)</th>
<th>Glu₁ (%)</th>
<th>Glu₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A253</td>
<td>14.4 ± 0.8*</td>
<td>8.0 ± 0.5 (55.6)*</td>
<td>90.7</td>
<td>9.3</td>
</tr>
<tr>
<td>FaDu</td>
<td>25.2 ± 0.2</td>
<td>9.0 ± 0.1 (36.5)</td>
<td>95.9</td>
<td>4.1</td>
</tr>
<tr>
<td>SQ₁</td>
<td>24.4 ± 0.6</td>
<td>14.7 ± 0.3 (60.2)</td>
<td>94.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Mean of three separate experiments ± SD.

** DISCUSSION **

Although MTX is considered to be an “active” drug in the treatment of patients with epidermoid carcinoma of the head and neck, only one third of patients treated have a transient objective response. Thus information on inherent or natural resistance to this agent, as well as acquired resistance, may be useful and lead to new therapeutic strategies. We selected three cell lines derived from patients not previously treated with MTX to investigate further mechanisms of inherent drug resistance. Growth inhibition studies showed a marked disparity in sensitivity to this drug when MTX exposure was 4 or 24 h; when exposures were extended to 72 h these differences were minimal (Fig. 1). We attempted to elucidate the causes for the different sensitivities to MTX observed, based upon the known mechanisms of acquired resistance to this drug.

A screening assay for MTX resistance, based upon in situ or whole-cell assay for thymidylate synthase, was first used. The results of this assay were consistent with the possibility that the relative resistance of the A253 and SQ₁ lines to MTX, as compared to FaDu, was due to poor retention of this drug, a finding now known to be a result of poor polyglutamylation of MTX (three or more glutamates) since MTX and MTX diglutamate (two glutamates) are known to efflux rapidly from cells when the extracellular concentration is low. Thus rapid recovery of in situ thymidylate synthase activity occurred when A253 and SQ₁ cells that were loaded with MTX were resuspended in drug-free medium. After excluding other possible causes of MTX resistance described (increased DHFR, altered DHFR with decreased affinity for MTX, decreased thymidylate synthase activity (37-40), and impaired uptake of MTX), polyglutamate formation was measured in cells incubated in 10 μM [³H]MTX for 4 and 24 h. A253 cells formed the least amount of MTX polyglutamates (three to six glutamates); SQ₁ cells were intermediate in their ability to form MTX polyglutamates, while FaDu cells contained almost 7 times as much MTX polyglutamates (three or more glutamates) as A253 cells and approximately twice the concentration of these compounds as the SQ₁ line.

Since impaired uptake could limit the intracellular concentration of MTX achieved and, therefore, free MTX (not bound...
to DHFR), it was important to attempt to distinguish between this possibility and impaired polyglutamylation as the cause for the low levels of intracellular polyglutamates found in the A253 line. In order to minimize the effect of polyglutamylation that would lead to increased retention and uptake of MTX, we studied the intracellular concentration of MTX after only 30 min of incubation, before long-chain MTX polyglutamates would be expected to form. Substantial amounts of free or unbound MTX were present in all of these lines, and a small amount of diglutamate formation was observed at this time. Less total MTX was found in the A253 line than in the FaDu or SQ lines. Thus, although uptake of MTX was less in the A253 line than in the other two cell lines, sufficient free MTX was present to enable MTX polyglutamates to be formed in all three cell lines. We conclude that defective polyglutamylation is the major cause of the A253 resistance to MTX, although poor uptake may also contribute to the low level of polyglutamylation observed in the A253 line. Of interest was the finding that, when FPGS activity was measured in extracts from the three cell lines, there were no appreciable differences. This is in contrast to a cell line described that was resistant to high dose 24-h exposures to MTX (41). Other causes for poor polyglutamylation observed in whole cells must be invoked to explain the results obtained, rather than a low level of this enzyme activity or an alteration of the enzyme leading to decreased MTX polyglutamate formation, as has been observed. A MTX-resistant breast cancer cell line, ZR-75, has been previously reported to also have impaired uptake of MTX, as well as decreased intracellular MTX polyglutamylation (42). The level of FPGS was slightly lower in the A253 line, as compared to the FaDu and SQ lines, but the significance of this relatively modest decrease in activity is not clear, especially when compared to the low activity of MTX polyglutamates in intact cells. Possible reasons for this low activity in intact cells are a decreased affinity of the enzyme for the other two cofactors required, MgATP and glutamate, or a low level of the glutamate substrate (43). An increased rate of breakdown of intracellular polyglutamates is also a possible explanation for these findings (44).

These data support the growing body of evidence that indicates that MTX is a “prodrug” and an important determinant of MTX cytotoxicity is polyglutamylation (26). The ability of cells to polyglutamate this drug is related in particular to toxicity to high pulse dose administration of the drug, whereas polyglutamylation appears to play less of a role in cytotoxicity when long term continuous exposure (72 h or longer) to the drug occurs (41).

It will be important to determine the causes for natural resistance to MTX in fresh tumor tissue from patients with head and neck cancer, perhaps utilizing the in situ thymidylate synthase screening test as described. Tumor cells showing poor polyglutamylation would then be candidates for constant infusion MTX over several days or for trimetrexate, a drug that has good activity in vitro and does not require polyglutamylation for retention.

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