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PROLIFERATION, MIGRATION AND INVASION OF HUMAN GLIOMA CELLS EXPOSED TO ANTIFOLATE DRUGS

A.J.A. TERZIS[1,3,4], T. FISKERSTRAND[2], H. REFSUM[2], P.M. UELAND[2], H. ARNOLD[1] and R. BJERKVIG[3]

[1]*Department of Neurosurgery, University of Lübeck, Ratzeburger Allee 160, 2400 Lübeck 1, Germany;* [2]*Department of Pharmacology and Toxicology; and* [3]*Department of Pathology, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway.*

The present study describes the effects of 2 folate antagonists, methotrexate (MTX) and the lipophilic antifolate trimetrexate (TMX) on 2 permanent human glioma cell lines (GaMg and D-54Mg) grown as monolayers and as multicellular tumor spheroids. In addition, the effects of drug exposure on tumor cell invasion was studied using a three-dimensional organ co-culture system. In monolayer cultures, TMX was a more potent inhibitor of cell growth than MTX, especially towards the GaMg cell line. The 2 drugs, however, showed similar cytotoxicity as assessed by the plating efficiency assay. Reduced ability of directional migration of cells on a plastic surface was seen by either antifolate usually at concentrations to 10-fold higher than those exerting a cytotoxic effect in the plating efficiency assay. TMX was somewhat more potent than MTX as an inhibitor of spheroid growth. When tumor spheroids were exposed to MTX or TMX at concentrations that caused 65 to 70% inhibition of cell migration, there was a latent period of 4 to 5 days before inhibition of spheroid growth ensued. Invasion was investigated in a co-culture system, where tumor spheroids were confronted with fetal rat brain cell aggregates. Neither drug reduced tumor cell invasion, although histological examination revealed toxic effects both in GaMg and in D-54Mg spheroids. We conclude that spheroids from human glioma cells were less sensitive to the antifolates than monolayers. For both drugs a latency period was observed before inhibition of spheroid growth. The spheroids retained their ability to invade normal brain tissue when exposed to levels of folate antagonists inhibiting spheroid growth.

Primary brain malignancies are highly invasive. This may account for the poor prognosis even after radical surgery. Although the tumor may appear macroscopically limited at operation, microscopical examination nearly always reveals infiltrating tumor cells in surrounding brain. Surgery in combination with radiotherapy are vital elements in therapy of such tumors, while chemotherapy has been more controversial (Paoletti *et al.,* 1990).

The central nervous system was long considered as a pharmacological sanctuary for most systemically delivered chemotherapeutic agents, and the view still prevails that properties such as lipid solubility and low molecular weight favour the distribution of drugs across the intact blood-brain barrier (Grieg, 1987). However, there is evidence that water-soluble cytostatic agents are able to penetrate into brain tumors (Rosner *et al.,* 1983), probably due to a disrupted blood-brain barrier. Thus, the lack of response to chemotherapy may be attributed to the biological characteristics of the tumor, including drug sensitivity at the cellular level and distribution barriers or metabolic cooperativity related to the 3-dimensional structure of the tumor.

The *in vitro* sensitivity of cancer cells towards cytotoxic agents is usually determined by clonogenic survival or growth inhibition of drug-treated cells cultured as monolayers or in suspension. However, the effect of the cytostatic agents may be modulated by their distribution into the tumor, the presence of avascular regions containing hypoxic cells (Carlsson and Nederman, 1992), metabolic adaptations and varying proliferation rate within the tumor (Sutherland, 1986).

It has been shown that spheroids both from normal and from malignant tissues may maintain several biochemical and morphological characteristics similar to those of the corresponding tissue *in vivo* (Bjerkvig *et al.,* 1992). Therefore, testing cytostatic agents on spheroids may provide additional information concerning drug effects *in vivo* (Carlsson and Nederman, 1992).

Trimetrexate (TMX) is active against a range of human and murine tumor cell lines, and shows a broader *in vitro* spectrum than methotrexate (MTX) (Lin and Bertino, 1991), which has been shown to be cytotoxic against a panel of human glioma cell lines (Mabuchi *et al.,* 1991). Both agents act by inhibiting the enzyme dihydrofolate reductase, and thereby block the regeneration of tetrahydrofolate from dihydrofolate and inhibit the folate-dependent synthesis of purine and thymidylate (Lin and Bertino, 1991). They differ with respect to cellular uptake and intracellular metabolism. MTX is taken up by the reduced folate transport system and is converted to and retained as polyglutamates in the intracellular compartment, whereas TMX seems to enter the cell primarily by passive diffusion, and is not subjected to polyglutamation (Lin and Bertino, 1991).

MTX is a useful agent for the treatment of acute leukemia and several solid tumors, but the clinical results against primary brain tumors have been variable (Shapiro, 1977). TMX has just entered clinical trial (Lin and Bertino, 1991), and one study showed no effect against anaplastic gliomas (Cairncross *et al.,* 1990). There are indications, both from experimental and from clinical studies, that MTX distributes into tumors located in the central nervous system, whereas data for TMX are limited to the demonstration of significant amounts of the drug in cerebrospinal fluid (Lin and Bertino, 1991). These data may suggest that both MTX and TMX gain access to gliomas.

Sparse data exist on the effect of MTX on spheroid cultures (Embleton *et al.,* 1991; West *et al.,* 1980), and TMX has not been tested in such systems. Furthermore, invasion in the presence of these antifolates has not been investigated. In the present study we describe the *in vitro* effects of MTX and TMX on spheroid growth and invasion, using 2 human glioma cell lines. These drugs were selected because their mechanisms of action and metabolic effects are similar, but their lipid solubility, cellular uptake and tissue distribution are different. The aim was to evaluate how the diverse properties of these drugs affected the tumor cells in various *in vitro* systems which may appropriately reflect tumor-cell behavior *in vivo.*

MATERIAL AND METHODS

Chemicals and drugs

MTX was obtained from Nycomed (Oslo, Norway), and TMX was kindly supplied by Parke-Davis (Warner-Lambert, Plymouth, UK). MTX and TMX were stored in dry form at −20°C. Before being added to the culture medium, MTX or TMX were serially diluted in PBS to the concentrations required. Osmium tetroxide and Epon 812 were from Fluka

[4]To whom correspondence and reprint requests should be addressed.

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(Buchs, Switzerland), and glutaraldehyde, Giemsa solution and toluidine blue were from Merck (Darmstadt, Germany).

Tumor cell lines

Monolayer and multicellular tumor spheroids from 2 human glioma cell lines were used. The human D-54Mg (passage number 60 to 70) cell line was kindly supplied by Dr. D.D. Bigner, Duke University Medical Center, Durham, NC (Bigner *et al.*, 1981). The GaMg (passage number 50 to 60) was obtained from a 42-year-old female and histologically identified as a glioblastoma (Akslen *et al.*, 1988).

Both cell lines were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated new-born bovine serum, 4 times the prescribed concentration of non-essential amino acids, 2% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were cultured at 37°C, in 100% relative humidity, 95% air and 5% CO_2.

Tumor spheroids

Tumor spheroids were initiated by the agar overlay culture method described by Yuhas *et al.* (1977). Briefly, spheroids were formed by seeding 5×10^6 cells in 20 ml growth medium into 80-cm^2 agar-coated tissue-culture flasks (Nunc, Roskilde, Denmark). After 10 days in culture, spheroids with diameters between 200 and 350 μm were selected for experiments.

Preparation and characteristics of brain aggregates

Fetal rat brain cell aggregates were prepared according to the procedure of Bjerkvig *et al.* (1986). Briefly, rat fetuses were removed at the 18th day of gestation. The brains were dissected out and the meninges removed. The brain tissue was then minced and serially trypsinized at 37°C. The resulting single-cell suspension was centrifuged and re-suspended in complete DMEM. The cells were seeded in 0.5 ml DMEM in 16-mm multiwell dishes previously coated with 0.5 ml of DMEM-agar. Two days later, the aggregates were transferred to a large number of wells and allowed to mature for 20 days. Such mature brain aggregates have a well-developed neuropil with mature neurons, astrocytes and oligodendrocytes. Myelinated axons and synaptic complexes are frequently observed in the aggregates, thus showing many similarities to the normal adult brain (Bjerkvig *et al.*, 1986).

Monolayer growth

For both cell lines, 10^4 cells in 2 ml of DMEM were seeded into 3.5-cm dishes (Nunc). Various concentrations (1 nM to 3 μM) of MTX or TMX were added to the cells 2 days after seeding. The duration of drug exposure was 24 hr and 96 hr.

Cells exposed for 24 hr were washed with PBS and new medium without drug was added. During the 96-hr drug exposure, the medium was not changed. Monolayer cultures exposed for 24 hr and 96 hr were harvested 96 hr after addition of drugs. The cell number was determined by using a Coulter Counter (Coulter Electronics, Luton, UK). The experiments were done in triplicate.

Plating efficiency

A standard plating efficiency assay was used to determine the cytotoxic effects of the drugs. From each cell line, 2×10^4 cells were transferred to 6-cm dishes (Nunc) containing 4 ml of DMEM. The drugs were added to the cells 2 days after seeding. Drug exposure was 24 hr and 96 hr, as described above. After exposure, the cells were counted and 200 cells were plated (6 parallels) into 6-cm dishes with 4 ml of medium. After an incubation period of 7 to 14 days, the colonies were washed in PBS, fixed in methanol and stained with a 10% Giemsa solution in PBS. The colonies, defined as 25 or more cells, were then counted. The experiments were performed in duplicate.

Tumor cell migration

Spheroids (diameter 200 to 350 μm) were placed in uncoated 16-mm multiwell dishes (Nunc) filled with 500 μl DMEM, and exposed to varying concentrations of MTX or TMX for 96 hr. Each group consisted of 4 spheroids. The spheroids plated within 2 hours, and the cellular outgrowth from one spheroid was defined as a colony. Colony diameters were measured regularly in the phase-contrast microscope over a 4-day period (96 hr), and the colony outgrowth areas were then determined. The experiments were done in triplicate.

Spheroid growth

For each experiment, 20 spheroids (diameter 200 to 350 μm) were transferred individually into 16-mm multiwell dishes (Nunc). The dishes were base-coated with 0.5 ml 0.75% DMEM-agar and filled with 1 ml of DMEM. The spheroids were divided into 5 groups, with 4 spheroids in each group. One group was used as control. Both the D-54Mg cells and the GaMg cells were exposed to 2 concentrations of either MTX or TMX, which were selected on the basis of inhibition of tumor-cell migration. After 96 hr exposure, the cells were washed with PBS and new medium without drug was added. The diameters of the spheroids were measured regularly in a phase-contrast microscope over a 15-day period and the spheroid volume was calculated. The experiments were done in triplicate.

Drug treatment of brain aggregates

Twenty aggregates (diameter 250 to 300 μm) were transferred to 16-mm multiwell dishes (Nunc). The dishes were base-coated with 0.5 ml 0.75% DMEM-agar and filled with 1.5 ml of DMEM. The brain aggregates were divided into 5 groups, with 4 aggregates in each group, and treated with MTX or TMX according to the same protocol as used for the spheroids, except that experimental period was reduced to 10 days. In this period, the diameters of the aggregates were measured regularly in a phase-contrast microscope and the aggregate volume was calculated. The experiments were done in duplicate.

Tumor-cell invasion

Prior to confrontation, the tumor spheroids were treated with MTX or TMX for 6 days (in order to obtain effective growth inhibition), according to the protocol described above for spheroid growth. There were 4 parallels in each of the 5 groups. Then the spheroids were transferred to 96-well multiwell dishes with a sterile Pasteur pipette and confronted with brain aggregates. The co-cultures were continuously exposed to MTX or TMX during a 4-day period, and then fixed for light-microscopic examination and morphometric analyses. The reduction of brain-aggregate volume as a percentage of the initial brain volume before confrontation was used to quantify the invasive process (see below). The experiments were done in duplicate.

Light microscopy

The co-cultures were fixed in 2% glutaraldehyde in 0.1 M sucrose-adjusted sodium-cacodylate buffer (300 ± 25 mOsm). After 24 hr, the specimens were washed in the same buffer, without glutaraldehyde, and post-fixed for 1 hr in 1% OsO_4 before serial dehydration in increasing gradients of ethanol up to 100%. Embedding of the co-cultures in Epon 812 was performed using graded mixtures of epon-propylenoxide. The specimens were polymerized for 48 hr at 60°C.

Morphometry

Cavalieri's principle for direct estimation of volume from systematically sampled sections was used to determine the amount of brain tissue remaining after co-culture with tumor-cell spheroids (Gundersen and Jensen, 1987). Serial semithin

sections (1.5 μm) were prepared from the specimens using a Reichert-Jung Microtome 2040 (Vienna, Austria). Every 15th section was sampled, starting with one of the 10 first sections, which was randomized. The sections were then stained with toluidine blue for light-microscopic examination. The brain tissue and the tumor tissue were easily distinguishable by the difference in morphology. The areas of tumor tissue and remaining brain tissue were then measured in each section by morphometry, using an Image Analysis system (Kontron, Eching, Germany). The area of tumor tissue and brain tissue measured on each slide, multiplied by the distance between every sampled section (15 × 1.5 μm) gave an estimate of the volume between the sampled sections. The total volume of tumor tissue and of remaining brain tissue was calculated by summation of all the individual volumes. The coefficient of error in this method for estimating volumes of sections is below 5% (Gundersen and Jensen, 1987).

RESULTS

Growth inhibition of cells cultured as monolayer

MTX and TMX inhibited growth of both D-54Mg and GaMg cells in a dose- and time-dependent manner. The cells were exposed to drug for 24 or 96 hr, and the dose-response curves were shifted to the left by increasing the duration of exposure (Fig. 1*a*,*b*).

MTX inhibited the growth of both cell lines to the same extent and similar dose-response curves were obtained, whereas TMX was more efficient towards the GaMg cells than towards D-54Mg cells, especially after prolonged (96 hr) drug exposure (Fig. 1*a*,*b*). Short (24 hr) exposure of D-54Mg cells to TMX produced a dose-response curve with 2 components (Fig. 1*a* (arrow)). Prolonging the exposure-time to 96 hr, this second component is not observed (Fig. 1*a*).

Cytotoxicity in monolayer culture

Cytotoxicity was assessed for both cell lines by plating efficiency after 24 and 96 hr of drug exposure. It is shown that TMX and MTX induced a dose- and time-dependent toxicity towards the D-54Mg and GaMg cell lines, and the 2 drugs were essentially equally effective, and showed the same potency towards both cell lines (Fig. 1*c*,*d*).

For the D-54Mg cells, the cytotoxicity curves for both MTX and TMX paralleled the curves for inhibition of growth rate. The fraction of the D-54Mg cells showing delayed growth inhibition (Fig. 1*a*) also showed delayed cytotoxicity, as indicated by 2 phases in the plating curve obtained at 24 hr of TMX exposure (Fig. 1*c*, arrow).

For the GaMg cells, the potency of MTX was about the same in the plating efficiency assay (Fig. 1*d*) and in the growth inhibition assay (Fig. 1*b*). In contrast, the cytotoxic effect of TMX against these cells (Fig. 1*d*) was observed at a 6-fold higher concentration than those inhibiting growth (Fig. 1*b*).

Tumor-cell migration

The spheroid outgrowth curves were determined for both cell lines exposed for 96 hr to increasing concentrations of MTX or TMX (Fig. 2).

For the D-54Mg cells, the outgrowth area was reduced by about 50% when continuously exposed to 30 nM of TMX during the 4-day treatment period. MTX was less potent, showing a similar reduction in colony area at 100 nM.

For the GaMg cells, a 50% reduction of outgrowth area was obtained by 30 nM for TMX and by 100 nM for MTX. For both

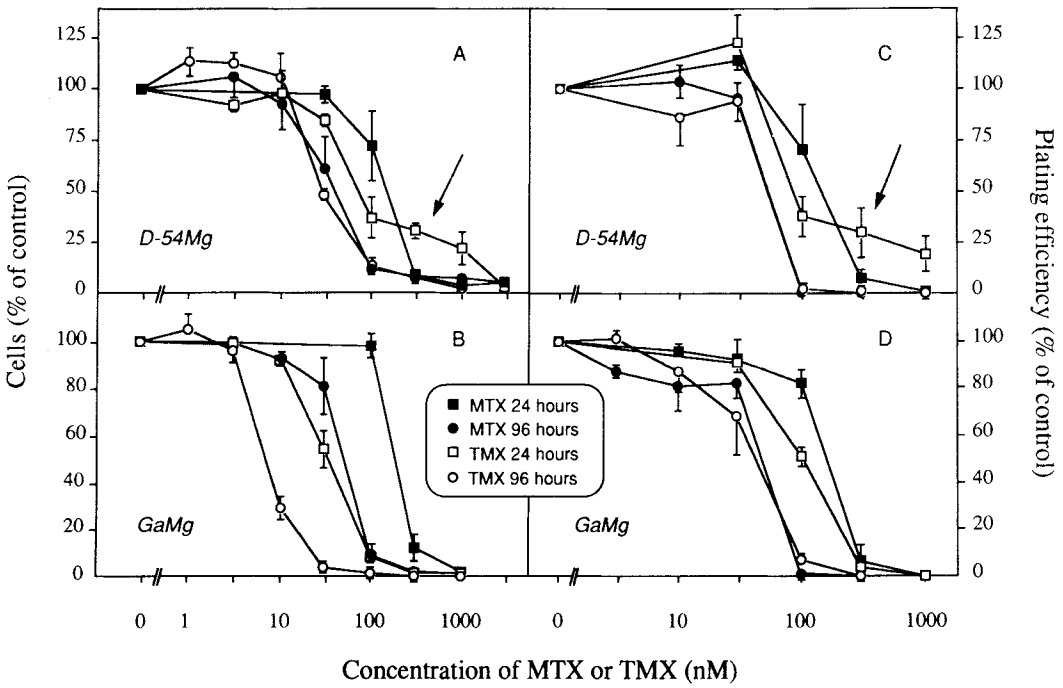

FIGURE 1 – Left panel. Monolayer growth inhibition of D-54Mg *(a)* and GaMg *(b)* cells after exposure to different concentrations of MTX and TMX for 24 or 96 hr. Results are expressed as total cell number as a percentage of control 96 hr after addition of drug. Each point represents the average of 3 experiments. Right panel. Relative plating efficiency of D-54Mg *(c)* and GaMg *(d)* cells after exposure to MTX and TMX for 24 or 96 hr. Plating efficiency is defined as the number of colonies as a percentage of control. Each point represent the average of 2 experiments with 6 parallel dishes in each experiment (bars = SEM). The arrows show the second phase of D-54 Mg drug sensitivity after 24 h exposure to TMX *(a,c)*.

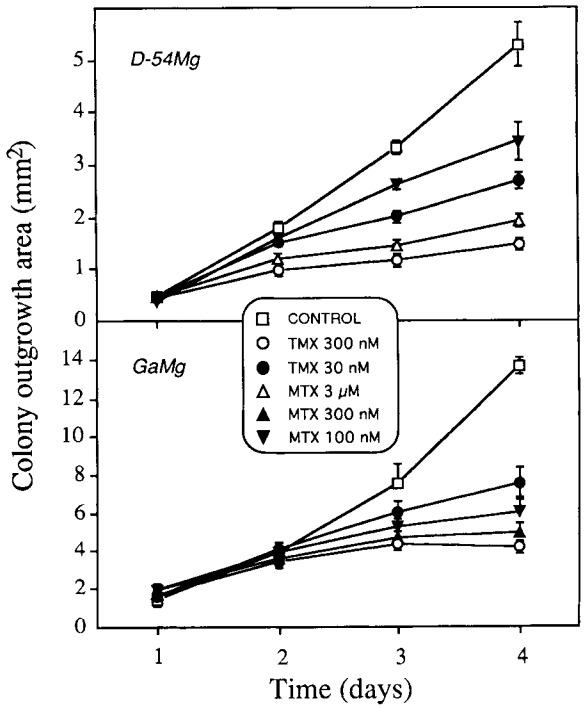

FIGURE 2 – Colony-outgrowth area from D-54Mg and GaMg spheroids treated with various concentrations of MTX and TMX for 96 hr. A dose-dependent reduction in outgrowth from the spheroids is seen. Each point represents the average of 3 experiments (bars = SEM).

drugs, 300 nM reduced the outgrowth area by 65 to 70% (Fig. 2).

Growth inhibition of spheroids

For both cell lines, spheroids continuously exposed to TMX and MTX showed a dose-dependent inhibition of growth which became apparent after 4 days of drug exposure (Fig. 3). In D-54Mg, TMX was more potent than MTX. An almost complete inhibition of spheroid growth was obtained at 300 nM TMX, while close to a 10-fold higher concentration of MTX was required to obtain a comparable inhibitory effect. In GaMg spheroids, TMX was only marginally more potent than MTX, and complete inhibition of growth was obtained at 300 nM of either drug between day 4 and 10. Treatment at lower drug concentrations indicated that the D-54Mg cells were more sensitive towards both drugs than the GaMg cells. After 10 days of drug exposure, some resumption of growth was observed, especially for the GaMg cells exposed to 300 nM MTX (Fig. 3).

At the doses of TMX and MTX given, no reduction in brain aggregate volume was observed (Fig. 3).

Tumor-cell invasion

In co-cultures without MTX or TMX, a marked replacement of brain tissue by invading glioma cells was observed (Fig. 4*a*,*d*). In the confrontation zone, the outer fibrous layer of glial cells was lost, and the brain-aggregate volume was reduced to 60 to 80% of the initial volume (Fig. 5).

Preliminary experiments showed that exposing tumor spheroids to the drugs for one day before and 4 days immediately after confrontation had no effect on tumor-cell invasion. We therefore decided to study whether completely growth-inhibited spheroids could invade normal brain tissue. In these

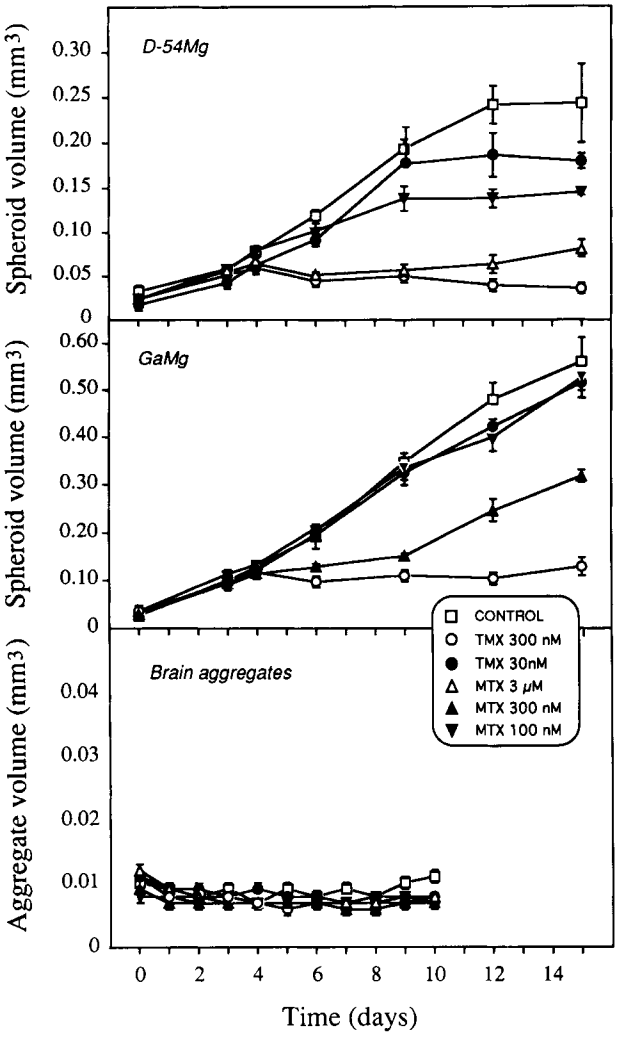

FIGURE 3 – Volume growth of D-54Mg (upper panel), GaMg spheroids (middle panel) and brain aggregates (lower panel) after treatment with various concentrations of MTX and TMX for 96 hr. Each point represents the average of 3 experiments (bars = SEM).

experiments, spheroids were pre-treated with MTX or TMX for 6 days, *i.e.*, a period long enough to induce growth arrest (Fig. 3), and for a further 4 days during confrontation. Notably, in these experiments, the same pattern of invasion was observed as for the confrontation experiments performed in the absence of drug (Fig. 4*b*,*c* and *e*,*f*).

After 4 days of confrontation, GaMg spheroids treated with 300 nM TMX showed a severe reduction in tumor volume as compared with control (without drug), and the brain aggregates were reduced to about the same extent as in the absence of drug, suggesting essentially the drug had no effect on the invasive process. Similar results were obtained with 30 nM of TMX and 300 nM and 100 nM of MTX, except that reduction in tumor volume was less pronounced (Fig. 5). These experiments were also performed with the D-54Mg spheroids. Again, neither MTX nor TMX affected the invasive process (Fig. 4).

Light-microscopic examination of the tumor tissue in the co-cultures exposed to MTX or TMX showed severe cell degeneration, with destruction and lysis of single cells. This was observed for both cell lines.

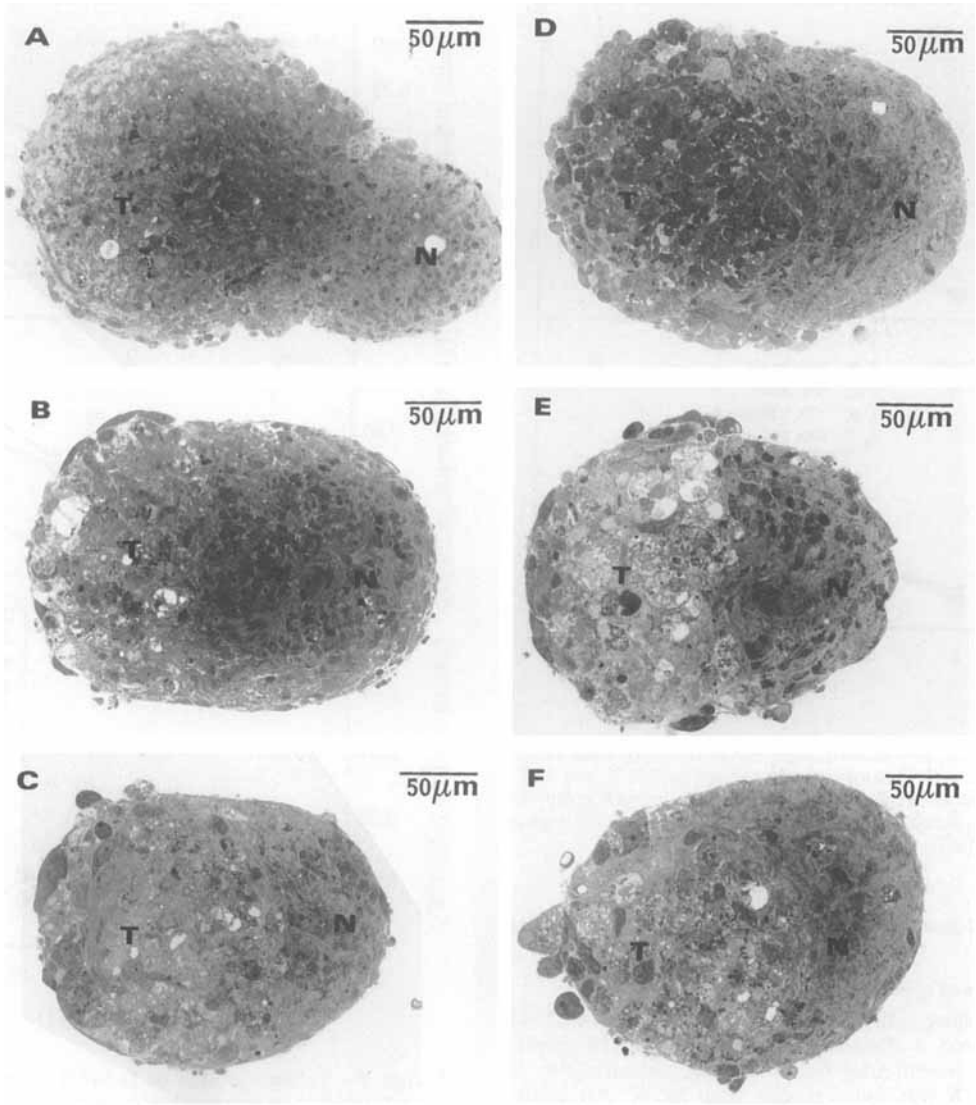

FIGURE 4 – Left panel. Photomicrographs (×280) of co-cultures between GaMg spheroids (T) and rat brain aggregates (N). *(a)* Control co-culture showing extensive invasion and degradation of brain tissue. GaMg spheroids treated with 300 nM TMX *(b)* and with 300 nM MTX *(c)* showed extensive invasion into the brain aggregates. Right panel. Photomicrographs (×280) of co-cultures between D-54Mg tumor spheroids and rat brain aggregates. *(d)* Control co-culture showing extensive invasion and degradation of brain tissue. Also D-54Mg spheroids treated with 300 nM TMX *(e)* and with 3 μM MTX *(f)* showed extensive invasion into the brain aggregates.

DISCUSSION

The disappointing results obtained with MTX (Shapiro, 1977) and TMX (Cairncross *et al.*, 1990) in the treatment of brain tumors can probably not be attributed only to the insufficient distribution of drug across the blood-brain barrier (Lin and Bertino, 1991). Intrinsic resistance of human glioma cells to antifolate drugs also seems an unlikely explanation since we (Figs. 1, 2) and others (Mabuchi *et al.*, 1991) have found that human glioma cell lines are sensitive towards antifolate drugs *in vitro*. Taken together, these data suggest that assessment of cytotoxicity of MTX and TMX against single glioma cells in culture could be an inappropriate model for studying drug effects *in vivo*. However, such experiments may provide data critical for the interpretation of experiments with multicellular systems such as cell aggregates and spheroids, and may suggest mechanisms of drug action at the cellular level.

MTX and TMX inhibit proliferation and colony-forming ability of both GaMg and D-54Mg cells in a dose- and time-dependent manner (Fig. 1). Thus, the human glioma cells show an intrinsic sensitivity towards TMX (Jackson *et al.*, 1988; Rhee *et al.*, 1990) and MTX which resembles that of several responsive murine and human cell lines, but which exceeds that of resistant cells and several human cell lines (Jackson *et al.*, 1988).

The high sensitivity of the GaMg cells towards TMX in the proliferation inhibition assay as compared with the clonogenic assay (Fig. 1) may be related to the higher proliferation rate of these cells as compared with the D-54Mg cells. Such disparity between drug-sensitivity assays has been previously reported both for MTX (Ueland *et al.*, 1986) and for TMX (Rhee *et al.*, 1990). It may be interpreted as resumption of clonogenic activity after transfer of inhibited cells to a fresh medium containing no drug, and may be explained by cellular leakage

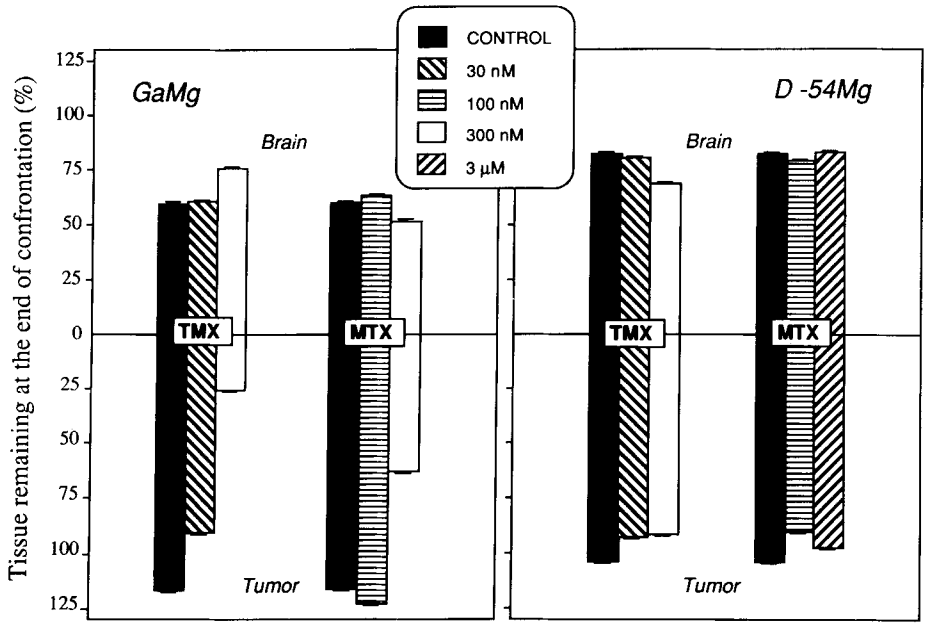

FIGURE 5 – Effects of MTX and TMX on invasion of D-54Mg and GaMg tumor spheroids. Volumes represent values calculated from reconstructed image analysis of serial sections. Four replicate cultures in 2 co-culture experiments (bars = SEM).

of TMX, which, in contrast to MTX, is not retained as polyglutamates (Jackson *et al.*, 1988). The short exposure of D-54 Mg cells to TMX (24 hr) produced dose-response curves with 2 components (Fig. 1*a,c* (arrows)), suggesting that a fraction of these cells was not completely inhibited, probably as a function of drug concentration and exposure time. On the other hand, a repair process could occur, since the cells were allowed to recover for a period of 72 hr.

The outgrowth assay measures the ability of the cells organized in a 3-dimensional structure to migrate and to proliferate. Cell migration is the dominating phenomenon during the first days after plating (Storme *et al.*, 1981). Spheroid growth, on the other hand, reflects proliferation of tumor cells. Notably, the effect of MTX and TMX on cell migration and spheroid growth shows several common features which are different from those observed for glioma cells in single-cell culture. First, TMX was a slightly more potent inhibitor than MTX of both migration and spheroid growth. Secondly, a higher concentration of antifolate drug (about 3 to 10 fold) was usually required to obtain inhibition of migration or inhibition of spheroid growth (Fig. 2). Similar observations have been made by Embleton *et al.* (1991), showing that osteosarcoma cells and colon-carcinoma cells cultured as monolayers were much more sensitive to MTX than spheroids. Analogous results have been obtained with several other cytostatic agents which are more effective against monolayers than the corresponding spheroids (Carlsson *et al.*, 1992).

Insufficient drug penetration in these 3-dimensional systems may reduce the effect of MTX. This is supported by the fact that the lipophilic agent TMX is more potent than MTX on spheroid growth; also, these agents have the same primary target enzyme, and a similar mechanism of action, and both are S-phase-specific compounds. An analogous observation has been made with lipophilic anthracycline analogues, which are more cytotoxic towards spheroid tumors than the water-soluble counterpart, adriamycin (Carlsson *et al.*, 1992). The importance of drug penetration is also shown in autoradiographic studies by West *et al.* (1980), showing limited MTX penetration into the central tumor mass of osteosarcoma spheroids.

The inhibiting effect of antifolates on spheroid migration was observed within 2 days. In contrast, when spheroids were exposed to MTX or TMX concentrations inhibiting migration by 75%, there was a latent period of 4 days before growth inhibition ensued (Fig. 3). The fact that a similar lag period was observed for both drugs suggests this delay in response is not only due to limited drug penetration into spheroids. Both MTX and TMX are S-phase-specific agents, and the cell-cycle distribution within the spheroids may affect growth inhibition (Carlsson *et al.*, 1992). We found that the GaMg cell line had a doubling time of 15 hr and 4 days in monolayer and spheroid culture respectively. The corresponding values for D-54Mg cells were 30 hr (monolayer) and 5 days (spheroids) (data not shown). The growth characteristics of the spheroids are thus more comparable with those observed *in vivo*, where the growth fraction is usually below 0.5 (Paoletti *et al.*, 1990), and the cell-cycle time is 70 to 80 hr (Paoletti *et al.*, 1990). This emphasizes the importance of using a relevant biological model when testing cytotoxic drugs, especially when the drug is a cell-cycle-phase-specific agent like the antifolates.

Local invasion into normal brain tissue is an inherent feature of malignant gliomas. Since both TMX and MTX extensively suppressed cell migration and growth (Figs. 2, 3), we studied whether these drugs affected invasion. Somewhat unexpectedly, neither drug inhibited invasion of the D-54Mg cells (Figs. 4, 5), despite the fact that the spheroids were pre-treated with drug and completely growth-inhibited at the time of confrontation. In GaMg co-cultures, high concentration of TMX (300 nM) markedly reduced the amount of remaining tumor tissue, and the brain aggregates were slightly better preserved. Notably, also, in these experiments, glioma cells extensively infiltrated the brain aggregates (Fig. 4).

The failure of cytotoxic concentrations of antifolate agents to inhibit invasion by tumor cells is an observation of considerable biological as well as clinical interest, and some explanations should be considered. The presence of differentiating

cells in the brain aggregates at the time of confrontation (Bjerkvig *et al.,* 1986) may be a source of factors affecting mitosis (Prehn, 1992) or migration (Lund-Johansen *et al.,* 1992). It is also conceivable that metabolic cooperativity exists, implying that the normal non-proliferative brain tissue furnishes nutritional components, such as folates, to the malignant neighbouring cancer cells.

It is concluded that MTX and TMX were essentially equally cytotoxic towards 2 human glioma cell lines cultured as monolayers. Our observations that migration and growth of glioma cells cultured as spheroids were less sensitive towards either drug emphasize the importance of drug penetration and altered cell-cycle kinetics as determinants of antifolate effects on solid brain tumors. Finally, the observation that glioma-cell invasion into normal brain tissue was virtually uninhibited, despite the presence of cytotoxic concentrations of either drug, is particularly interesting in relation to the suitability of spheroids as an appropriate model for studying anti-tumor effects *in vivo.*

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