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Microcinematographic Study on the Effect of Methotrexate upon Mouse Mammary Tumor Cells (MMT Cell Line)

Y. Alvarez-Rodríguez, Alicia Ruano, and Y. Valladares

Department of Biology and Biochemistry of Cancer*, Instituto Nacional de Oncología, Ciudad Universitaria, Madrid 3, Spain

Summary. *The effect of methotrexate (amethopterin) upon the MMT cell line was studied by time-lapse microcinematography, the plasma levels obtained after systemic administration of maximum tolerated doses of the drug in man being simulated in vitro. Cells in the logarithmic growth phase (large growth fraction population) were widely affected, although enough drug-resistant cells remained to regenerate the cell colony. Cells in the preconfluent growth phase (small growth fraction population) were less effected, because many cells were arrested at the G_0-phase, ouside the cell cycle. A drug-resistant colony always developed, making the drug therapy useless. The experiments showed that rescue treatment with leucovorin (citrovorum factor of folinic acid) was not effective either, because, at least on our experimental conditions, recovery of the mitotic activity was more rapid and the number of degenerating cells smaller with rescue treatment than with the conventional treatment. The results also suggested a new mechanism of methotrexate action in addition to the classic one of folic acid inhibition, which might consist in the inhibition of the production of formyl-methionyl-tRNA, part of the initiation complex in protein biosynthesis.*

Introduction

Chemotherapy currently has an important place in the treatment of cancer. For a better understanding of the mechanisms of action of the chemotherapeutic agents employed in cancer treatment it is necessary to combine pharmacological studies with knowledge of cell population kinetics [7, 8], which involves familiarity with the cell cycle [5, 6].

Two major problems in cancer chemotherapy are the high toxicity of the drugs for the normal cells at doses effective against neoplastic cells, and the development of drug resistance by cancer cells. Neoplastic cells are chromosomally heterogeneous, they have a high capacity for genetic adaptation, and resistant cells leading to resistant clones generally arise from the first contact between the neoplastic population and the chemotherapeutic agents.

This paper reports the effect of methotrexate (MTX; amethopterin) on MMT cells derived from a cell line stablished from a mouse mammary tumor by J. Sykes in 1962. For the preliminary toxicity studies HeLa cells, obtained from a human uterine cervix carcinoma in 1951 [3], were also employed.

Materials and Methods

Serial subcultures of MMT cells were made. The cells of a previous monolayer were detached with a 0.2% trypsin solution, centrifuged at 90 *g*, resuspended in Eagle's minimum essential medium supplemented with 10% calf serum and 1% nonessential amino acids, 100,000 cells per milliliter inoculated with a homogeneous suspension, and incubated at 37° C until cell confluence, when the next passage was made.

As a comparison, HeLa cell cultures were studied when indicated. The characteristics of the cell cycle were determined for both MMT and HeLa cells. The technique of plotting the percentage of mitoses labeled against time after pulse-labeling with tritiated thymidine [7] was used to calculate the phases of the cell cycle, and time-lapse microcinematography was utilized to calculate generation time and M-phase duration.

For time-lapse microcinematography study, Plus X-negative type 7231 or Eastman color negative type 7247 films and a Nikon model M inverted microscope furnished with an incubating chamber at 37 ± 0.1° C, a 16-mm Paillard Bolex motion-picture camera, and an intervalometer were used. In most instances the interval timer was set for exposure of one frame every 4 min; in this way 1 min of normal-speed projection at 24 frames per second corresponded to 4 days of real time.

* Coordinated center of the Consejo Superior de Investigaciones Científicas, Madrid

Reprint requests should be addressed to: Y. Valladares

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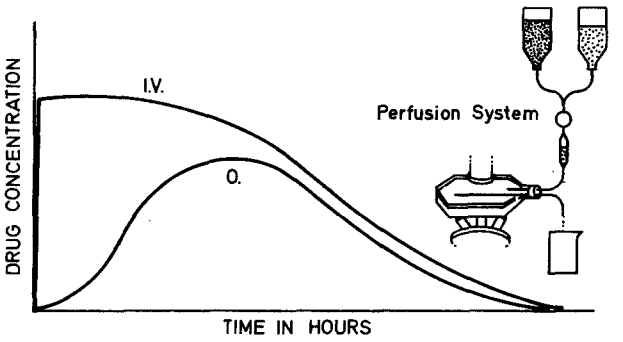

Fig. 1. Perfusion system for the in vitro administration of MTX at doses equivalent to the plasma levels. I. V., intravenous; O, oral

A perfusion system was arranged, allowing addition of MTX to the cell cultures at doses simulating the body plasma levels (Fig. 1). Cultures were prepared in Earle's T-15 flasks (15 cm^2 culture surface) with a silicone stopper perforated by two cannulas, an afferent one connnected to the continuous perfusion system, and an efferent one connected to a sterile flask for the liquid coming out. The perfusion system consisted of two flasks connected by a three-way valve. One of the flasks contained growth medium and the other the MTX solution prepared in growth medium. The liquid flow was controlled by means of a clamp on the rubber tube. Concentration and elimination of the assayed drugs were controlled by means of an LKB 11300 Ultrograd gradient mixer and an LKB 2115 Multi-Perpex peristaltic pump. T-15 flasks were inoculated with 7 ml cell suspension containing 100,000 cells/ml.

All materials having contact with the growth medium flow were previously assayed to confirm the absence of nonspecific cytotoxic effects. The tube entering the culture flask was arranged in a long-run coil through a water bath kept at 37° C within the incubation chamber.

When necessary, cells were fixed in Bouin's fluid, stained with hematoxylin and eosin, and mounted in Canada Balsam.

Methotrexate Toxicity upon MMT and HeLa Cells

A. Preliminary Studies. Preliminary experiments were done to assay the toxicity of MTX at several concentrations on MMT and HeLa cells. Observations were made at two different periods during cell growth: the logarithmic phase, simulating a tumor having a large growth fraction, and the preconfluent phase, to simulate a cell population with a small growth fraction, taking advantage of the contact inhibition of mitosis produced when cells join one another. Ten milliliters and 100,000 cells/ml were inoculated into Corning plastic flasks with 25 cm^2 ceulture surface. Cell viability was previously calculated by exclusion staining of an aliquot with erythrosin B. Cultures were incubated at 38° C, and growth medium changed every 24 h; 0.5, 5.0, 10.0, 25.0, and 50.0 μg MTX/ml were added to different flasks about 24 h after inoculation, coinciding with the first part of the logarithmic growth phase (large growth fraction), and about 96 h after inoculation, when the cultures were in the preconfluent phase (small growth fraction).

Pure MTX and leucovorin (citrovorum factor or folinic acid) were used, prepared for us by the Lederle Laboratories (Cyanamid Ibérica, S.A.) without the methylparaben and propylparaben usually employed for preservation of the pharmaceutical preparations. All solutions were freshly prepared before use. A concentrated solution was first made in GKN solution at pH 7.2, and this was used to prepare MTX at different concentrations in Eagle's minimum essential medium supplemented with 1% nonessential amino acids and 10% calf serum; pH was strictly maintained between 7.0 and 7.4, because at more basic pH values all the cells died within a few minutes.

Twenty-four hours after the antiblastic was added, it was removed by decanting and washing out the cultures with growth medium. Fresh growth medium was then added, and the cultures were incubated at 37° C, the medium being changed every 24 h. After 5 days a series of cultures was fixed and stained. Cells were washed in lukewarm GKN solution, decanted, fixed and stained. Cells were washed in lukewarm GKN solution, decanted, fixed in Bouin's fluid for 2 h at room temperature or 24 h at 4° C, washed in 70° C ethanol, and left to stand in 70° C ethanol for 24 h. Cells were then washed in 96° C ethanol for 1 min, washed in water, stained in Caracci's hematoxylin for 1 min, washed in tap water, dipped in distilled water for 5 min, stained in eosin for 10 s, washed in water, dehydrated in two washes of 96° C ethanol for 1 min each, air-dried, mounted on Canada balsam or glycerol, and observed.

Parallel series of cultures were maintained after 5 days, the medium being changed every other day. The time needed for monolayer confluence to occur or the number of groups of surviving cells 10 days after treatment was recorded. The cells we call resistant are those originating from the surviving cells. Resistant cells from MMT cell cultures treated with 25 μg MTX/ml during the logarithmic growth were subcultured twice to stimulate their growth, and then exposed again to MTX in an identical ways.

B. Time-lapse Microcinematography Study. Wild MMT cells and resistant MMT cells (MMT-R) were prepared for time-lapse microcinematography as previously indicated. When the cultures reached the logarithmic growth phase they were exposed to the following treatments:

a) Rapid perfusion with fresh medium containing 20 μg MTX/ml, followed by fresh medium without the drug at a continuous flow of 3.15 ml/h. As the liquid volume (7 ml) within the flask remained constant, the concentration of the drug diminished by half every 2 h.

b) Rapid perfusion with fresh medium containing 1500 μg MTX/ml, followed 2 h later by 1200 μg leucovorin, added rapidly to obtain 150 μg/ml final concentration, and then fresh medium without any drug at a continuous flow of 3.5 ml/h to diminish the concentrations of MTX and leucovorin by half every 2 h. After 7 h the perfusion fluid was supplemented with 15 μg leucovorin/ml until the end of the experiment.

With these procedures, we tried to simulate the plasma levels attained when maximum tolerated doses of MTX are administered to man. From the data reviewed by Bleyer [1], these doses vary between 80 and 900 mg/m^2 for conventional treatments, and between 900 and 1,800 mg/m^2 for the rescue treatment. We also simulated an average drug elimination of 2 h, considering the second-phase half-life elimination time for plasma calculated for MTX-treated patients by Pratt et al. [10]. For rescue treatment we simulated doses slightly higher than those recommended in the 'therapeutic guide to high-dose MTX therapy with citrovorum rescue' mentioned in Bleyer's work.

Results

Methotrexate Toxicity upon MMT and HeLa Cells

A. Preliminary Studies. From the first experiments carried out to study the in vitro cytotoxicity upon MMT and HeLa cells, we observed a large amount of detached cells and a more basic pH than in the control cultures as early as 24 h after MTX suppression. Both effects were more

Table 1. Preliminary studies on the effect of MTX upon MMT, MMT-R, and HeLa cell lines

Cells	MTX (μg/ml)	24 h exposure to MTX starting 24 h after flask inoculation (large growth fraction)		24 h exposure to MTX starting 96 h after flask inoculation (small growth fraction)	
		Groups of cells surviving per 25 cm^2 culture surface 10 days after exposure[a]	Days before monolayer became confluent after exposure	Number of cells per microscopic field 5 days after treatment[b]	Days passed before monolayer became confluent after exposure
HeLa	0	Innumerable	3	> 400	0
	0.5	Innumerable	6	> 400	4
	5	825	> 10	> 400	5
	10	625	> 10	170	7
	25	100	> 10	140	8
	50	4	> 10	96	9
MMT	0	Innumerable	3	> 300	0
	0.5	Innumerable	6	> 300	4
	5	975	8	> 300	4
	10	750	> 10	210	6
	25	150	> 10	160	7
	50	3	> 10	89	8
MMT-R	0	Innumerable	3	> 300	0
	0.5	Innumerable	5	> 300	3
	5	1075	7	> 300	4
	10	825	10	300	5
	25	200	> 10	140	7
	50	5	> 10	95	8

[a] Average from three culture flasks
[b] Average obtained by counting six microscopic fields of two different flasks

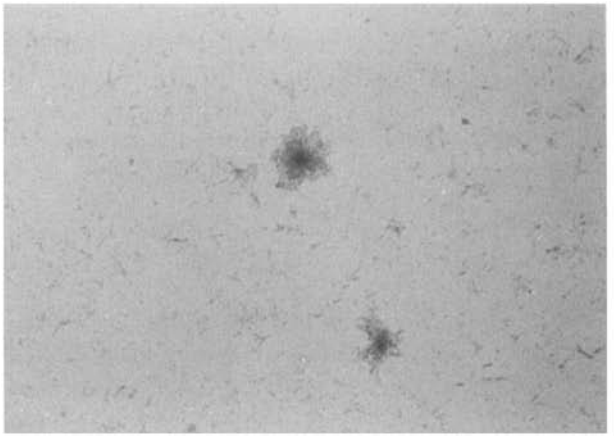

Fig. 2. Effect of MTX on logarithmically growing wild MMT cells, equivalent to a population having a large growth fraction. There is almost complete destruction of cells 5 days after exposure to 25 μg MTX/ml, although resistant cells always appear, originating drug-resistant clones; two are seen in this picture

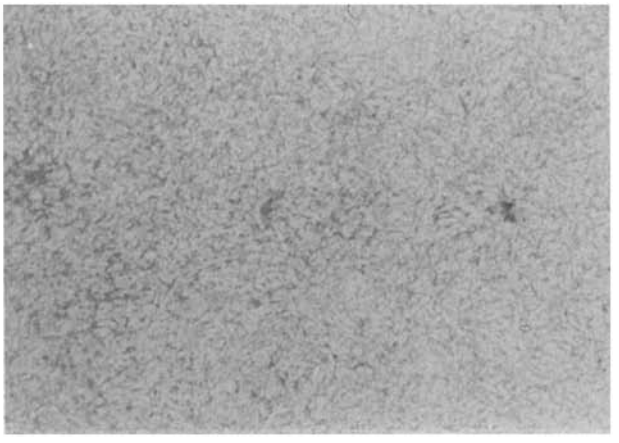

Fig. 3. Effect of MTX on the preconfluent growth phase of wild MMT cells, corresponding to a colony having a small growth fraction. Cell mortality is much lower, because cells that are not in cycle are not affected. The photomicrograph was obtained 5 days after addition of 25 μg MTX/ml

marked in cultures receiving higher doses of the drug. Table 1 shows some of the effects observed. Five days after treatment, the cells receiving MTX during the logarithmic growth phase (large growth fraction) showed wide plots of the culture flask surface cleared of cells, residues of degenerated cells, and dispersed groups of surviving cells (Fig. 2). The size of these groups increased during subsequent days; however, after 10 days none of the cultures receiving more than 10 μg MTX/ml produced confluent monolayers.

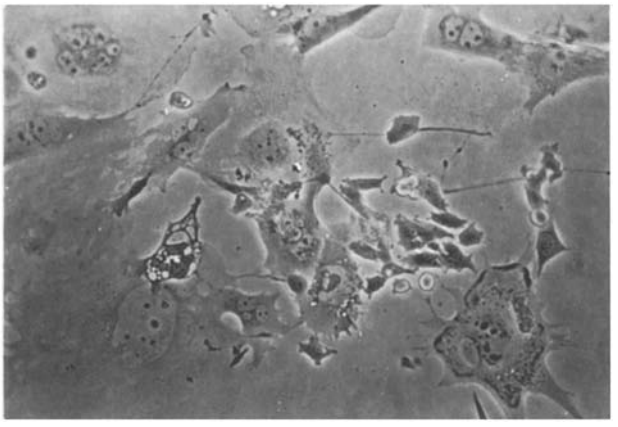

Fig. 4. Aspect of the MMT cell colony 7 days after exposure to 50 μg MTX/ml, added during the confluent phase. For details see text

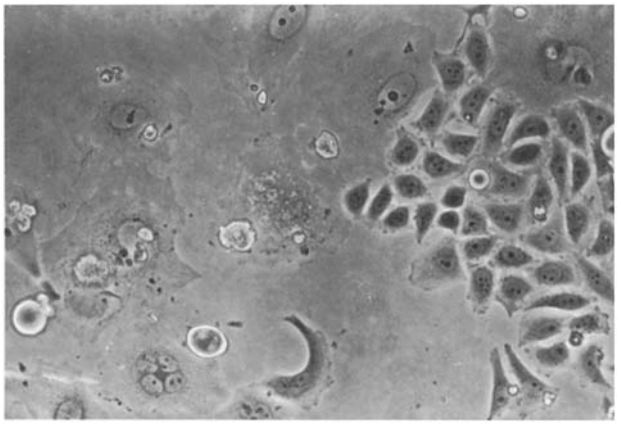

Fig. 5. Photomicrograph of the HeLa cell colony 9 days after exposure to 50 μg MTX/ml, added during the confluent phase. For details see text

Five days after MTX treatment the cell cultures receiving the drug during the confluent phase (small growth fraction) showed a marked decrease in cell number with doses higher than 10 μg/ml (Table 1, Fig. 3). All cultures became confluent again before 10 days after MTX treatment. However, the morphology of the monolayer was very much changed in both MMT and HeLa cells (Fig. 4 and 5); the main abnormalities observed were cells having large vacuoles, flat giant cells, and multinucleated cells, alternating with groups of cells that appeared normal.

Abnormal cells disappeared in MMT-R cells from the first subculture. For this reason the morphology of MMT and MMT-R was identical except in the actual culture originally exposed to the drug.

B. Time-lapse Microcinematography Study. Figure 6 shows the individual film frame analysis of mitotic activity for MMT and MMT-R cells growing in normal growth medium. Cell degeneration was not observed during the interval studied. The highest mitotic activity was recorded between 24 and 34 h after inoculation. Significant differences in the growth rhythm, phase M duration, and generation time were not observed.

From immediately after addition of the drug, MMT and MMT-R cells exposed to MTX during the logarithmic growth phase showed a diminution in cell movements and a flattening of the cells on the flask surface. Figures 7 and 8 correspond to the individual film-frame analysis of mitotic activity and cell degeneration. Mitotic activity decreased very soon after MTX was added; cell degeneration started after 12 h in MMT cells and 14 h in MMT-R cells, subsequently proceeding without interruption for 61 h in MMT cells and 58 h in MMT-R cells. Degenerating cells rounded off, detached partially from the growing surface, and died within the next 40 min. In both

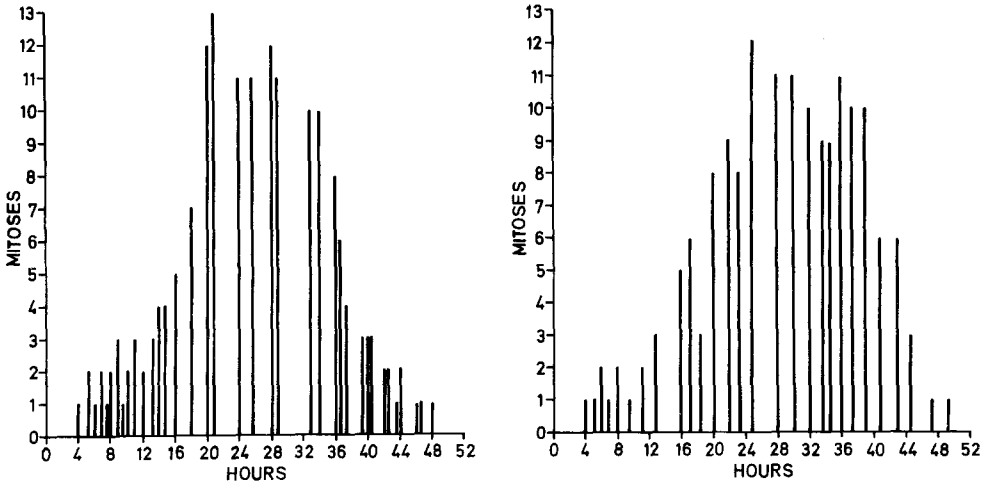

Fig. 6. Evolution of control MMT and MMT-R cells as studied by individual film frame analysis. No cell degeneration was observed

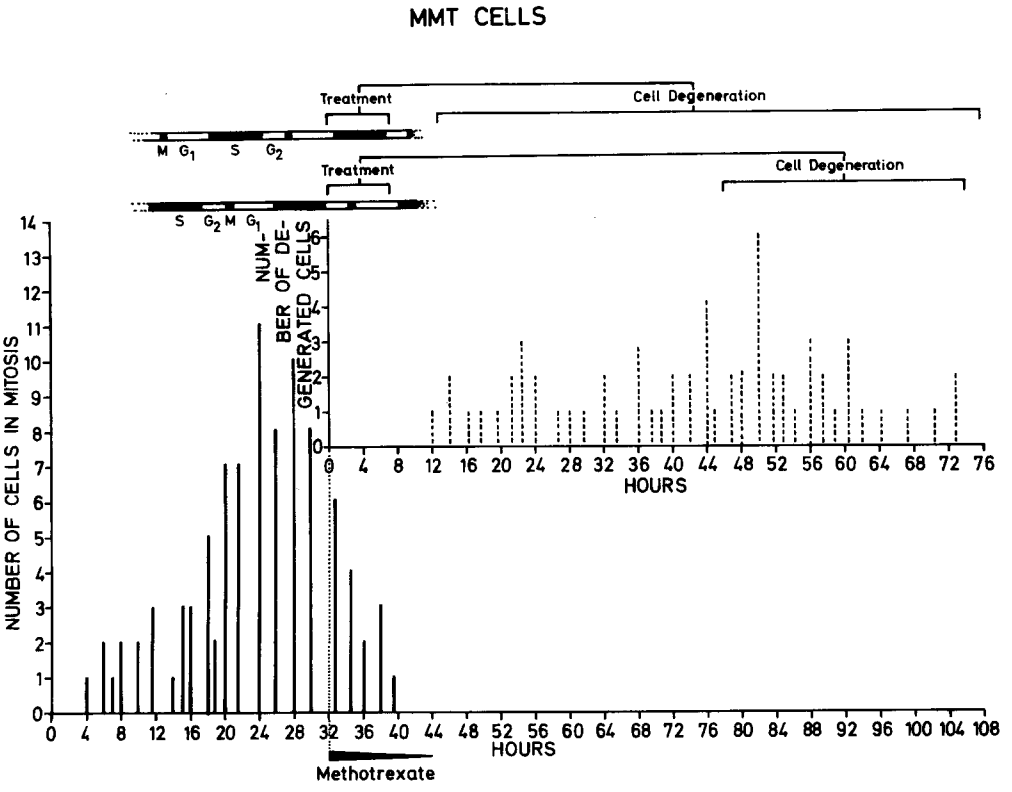

Fig. 7. Individual film frame analysis of mitotic activity and cell degeneration in MMT cells under the effect of MTX. For details see text

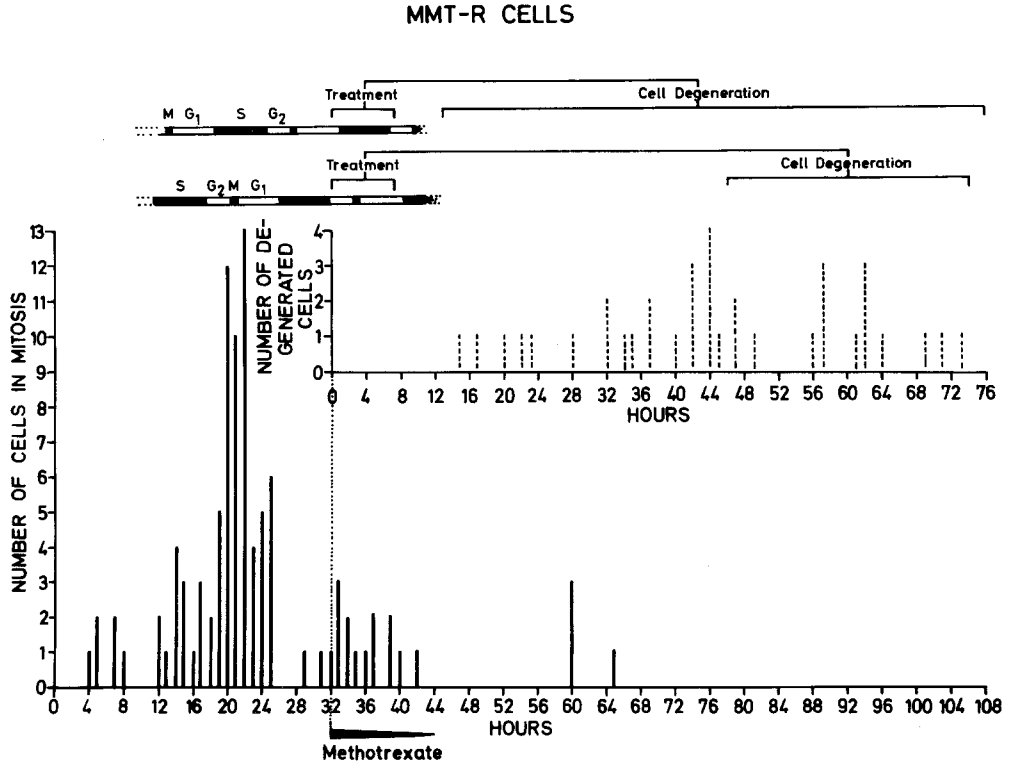

Fig. 8. Individual film frame analysis of mitotic activity and cell degeneration in MMT-R cells under the effect of MTX. For details see text

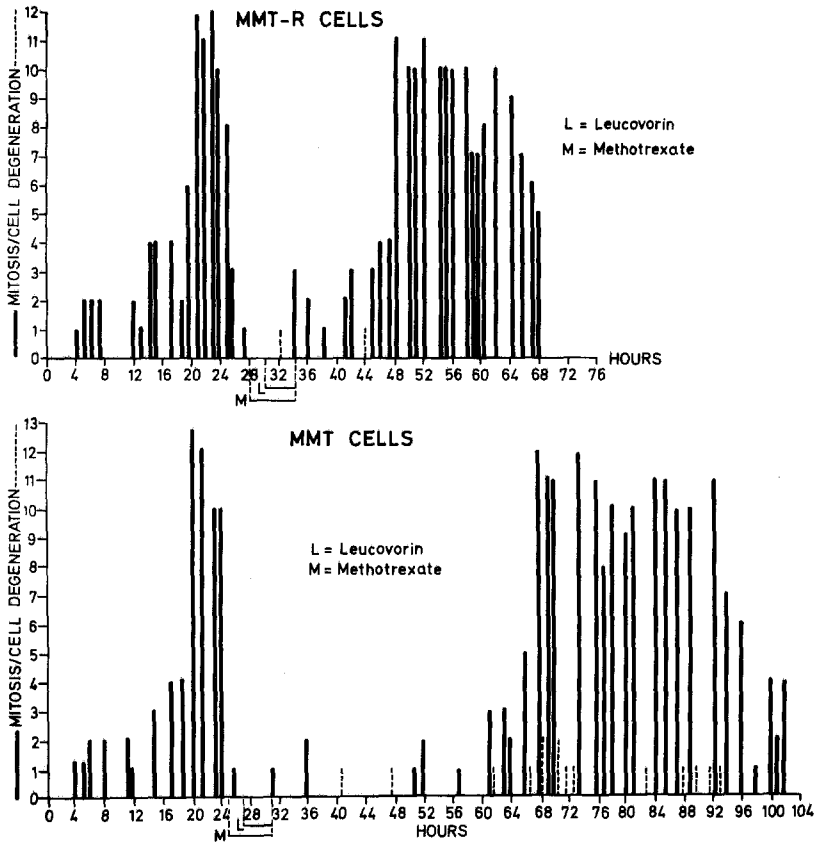

Fig. 9. Individual film frame analysis of the effect of leucovorin rescue upon MMT cells treated for the first time with MTX, and upon MMT-R cells. L, leucovorin; M, MTX

MMT and MMT-R cells MTX-induced diminution of mitotic activity was produced during the cell cycle exposed to it or during the next cell cycle. As for cell degeneration, many cells that had been irreversibly affected entered into proliferative rest and started degenerating 4–5 days after MTX treatment, a period equivalent to 6–10 times the generation time.

Cell degeneration and cell death affected 46% of the MMT cells, and 30% of the MMT-R cells that were present when first exposed to the drug. The cell population of the cultures finally regenerated, starting with a few cells that had been affected in a reversible way. However, recovery was not evident in the experiments described, because the probability of filming a repopulation group is very low: not only is the microscopic filming area always very small, but in addition the repopulation cell groups in the culture were very few at that moment (Fig. 2 and Table 1).

By correlating time of mitosis, moment of the cell cycle when the cells were exposed to the drug, and degeneration time, we observed that cells receiving MTX over most of the S-phase were blocked at the end of the S-phase or at the beginning of the G2-phase (large premitotic nuclei). Further cell degeneration took place and degenerating cells were dispersed, as shown in Fig. 7 and 8.

An interesting paradoxical effect was observed, consisting in the fact that most cells exposed to MTX during the G2- and the next G1-phase, but not during the S-phase, became arrested at the end of G1 or the beginning of the S-phase (small postmitotic nuclei), and cell degeneration was significantly delayed (Fig. 7 and 8). In the experiment reproduced in the graph, the late effect might be attributable to the presence of low levels of MTX during the first part of S. However, comparative experiments with complete removal of the drug 6 h after exposure led to the same results.

Figure 9 corresponds to the individual film frame analysis af MMT and MMT-R exposed to the second modality of treatment indicated under Materials and Methods, simulating the leucovorin rescue introduced by Goldin [4]. Mitotic activity recovered much earlier in MMT-R cells. Many more degenerating cells were found among MMT than among MMT-R cells. Cell degeneration and death was 10% for MMT and 1.7% for MMT-R, in relation to the number of cells existing when MTX was added. However, the final recovery was thorough in the filmed microscopic field and throughout the culture surface of the flask.

Discussion

The preliminary studies on toxicity upon MMT and HeLa cells described under Material and Methods and Results

showed: (a) a dose-dependent cytotoxic effect when 0.5–50 μg MTX/ml was used; (b) a more marked cytotoxicity in logarithmically growing than in preconfluent populations (Figs. 2 and 3); (c) a similar sensitivity of MMT and HeLa cells to the action of the chemotherapeutic agent when both were kept under the same experimental conditions; (d) the appearance of groups of drug-resistant cells in every case, including those where high doses of the anticancer agent were employed.

Cells that arose from cells surviving after a cycle of treatment (MMT-R cells) did not show any difference from wild MMT cells in their morphology, growth rhythm, or M-phase duration. However, MMT-R cells were less sensitive than MMT cells to a second MTX treatment.

The differences observed in both MMT and MMT-R cells do not support the idea of a genetically transmitted resistance characteristic for all MMT-R cells originating from originally resistant cells. A large part of MMT-R cells responded to MTX by reproducing the same phenomena as wild MMT cells. We therefore consider that wild cells are composed of a mostly susceptible population containing a variable number of cells that are able to originate clones of resistant cells. After several generations, populations evolving from originally drug-resistant cells consist in a mixture of individual cells having every degree of sensitivity [9].

Time-lapse microcinematography studies showed an inhibitory effect of MTX upon mitotic activity. We confirmed that MTX arrests the cell at the end of the S-phase or in the G2-phase. That MTX has a cell cycle S-phase-specific action interfering with mitosis is a proven fact; MTX binds very easily with the enzyme dihydrofolic acid reductase, and prevents the reduction of folic acid to tetrahydrofolic acid, which produces co-enzymes necessary for the synthesis of DNA via the reaction catalyzed by thymidilate synthetase transforming deoxyuridylic acid in thymidylic acid [2].

However, we observed that cells receiving MTX during the G2- and advanced G1-phase, but not during the S-phase, became arrested at the end of G1 or the beginning of S. In addition, we found a curious action requiring another explanation than that resting on prevention of the synthesis of tetrahydrofolic acid. This action was characterized by irreversible cell damage producing delayed death, some cells entering into proliferative rest and degenerating 4–5 days later. We attribute this effect to the existence of new points of attack of the drug. Among the important pathways of cell metabolism is that related to the conversion of methionyl-tRNA in formyl-methionyl-tRNA (Fig. 10). Tetrahydrofolic acid deficiency hinders formyl-methionyl-$tRNA_f$ formation, a substance forming part of the initiation complex in protein synthesis at the ribosomal station. If we assume that MTX acts at this level, we can explain our observation that high doses of the drug

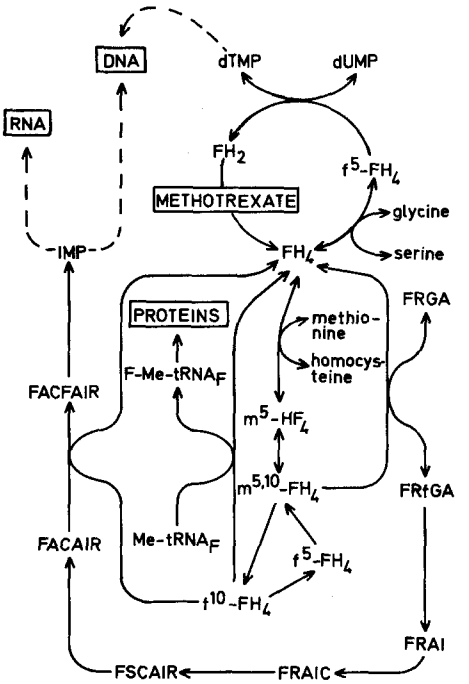

Fig. 10. Metabolic pathways of the reactions related with the folic acid cycle. DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dUMP, deoxyuridylic acid (deoxyuridine-5′-phosphate); TMP, thymidylic acid (thymidine-5′-phosphate); IMP, inosinic acid (inosine-5′-phosphate); FH_2, dihydrofolic acid; f^5-FH_4, N^5-formyl-tetrahydrofolic acid; FH_4, tetrahydrofolic acid; m^5-HF_4, N^5-methyl-tetrahydrofolic acid; $m^{5,10}$-FH_4, N^{5}-N^{10}-methylene-tetrahydrofolic acid; f^{10}-FH_4, N^{10}-formyl-tetrahydrofolic acid; Me-$tRNA_F$, methionyl-$tRna_F$; F-Me-$tRNA_F$, formylmethionyl-$tRNA_F$; FRGA, 5′-phosphoribosylglycinamide; FRfGA, 5′-phosphoribosyl-*N*-formylglycinamide; FRAI, 5′-phosphoribosyl-5-aminoimidazole; FRAIC, 5′-phosphoribosyl-5-aminoimidazole-4-carboxylic acid; FSCAIR, 5′-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole; FACAIR, 5′-phosphoribosyl-4-carboxamide-5-aminoimidazole; FACFAIR, 5′-phosphoribosyl-4-carboxamide-5-formamidoimidazole

inhibited cell growth, acting on the G1 period. This effect cannot be ascribed to protein synthesis inhibition concomitant with a decrease in serine or homocysteine, because the growth medium contains a sufficient concentration of these amino acids. Methotrexate led the cells into G_0, where they stayed for a time equivalent to several generation times before initiating its degeneration. In addition, the new attacking point would make it easier to explain the mechanism of action of leucovorin, considering that this compound, or N^5-formyltetrahydrofolic acid, is converted into N^{10}-formyltetrahydrofolic acid, directly or through the N^5-N^{10}-methylentetrahydrofolic acid, which is necessary for formyl-methionyl-$tRNA_f$ formation. This metabolic pathway, which is able to inhibit protein synthesis, is shorter than the path employed to convert N^5-N^{10}-methylene-tetrahydrofolic acid into N^5-methyltetrahydrofolic acid and tetrahydrofolic acid, a pathway involved in the passage of deoxyuridilic acid to deoxythymidylic

acid. On the other hand, cells arrested reversibly at the beginning of the S-phase by deoxythymidylic acid depletion recover when thymidine is added, which the cell converts into deoxythymidylic acid. It seems evident that if formation of one of the essential factors of the initiation complex at the level of the translation of the genetic message is lacking, it may produce irreversible damage, considering that the different kinds of mRNA, which are synthesized following a discontinuous pattern along the cell cycle, and are short-lived, do not find the necessary factors to initiate the translation process, so hindering the biosynthesis of enzymes. Finally, the amount of MTX needed to stop nucleic acid synthesis is higher than that necessary to block dihydrofolate reductase. For the present, the possibility of the said mechanism of action remains a working hypothesis based on indirect data; we have initiated some experiments to give our statement a more solid basis.

The results of the treatment with MTX alone simulating the levels attained by the administration of maximum tolerated doses to human patients showed that no more than a moderate destruction of neoplastic cells was accomplished. The effect was more marked in populations having a large growth fraction, and also in populations that had never been exposed to the drug. In all experiments enough neoplastic cells were left that had been not destroyed but only transiently inhibited, and these were able to regenerate the cell colony to completion.

The results of the rescue treatment with MTX and leucovorin, as indicated in the text, demonstrated that cell destruction was significantly less than with MTX alone, and the recovery of surviving neoplastic cells to repopulate the monolayer was more rapid. These differences were more marked for those cells deriving from cell populations previously treated with MTX.

If our experimental results can be directly extrapolated to human therapy, MTX monochemotherapy is not effective. We can think of activity of the drug only if it is metabolically activated within the organism or its combination with other drugs adds something to its action. Our experiments also showed that MTX alone is more active than in the rescue treatment with leucovorin.

Many factors not considered here could be involved in our results, such as a greater susceptibility of cells cultured in vitro or a larger diversion of the drug by nonspecific protein binding.

The results obtained, however, could lead to new ideas for more efficient use of MTX.

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