EFFECT OF METHOTREXATE CONCENTRATION AND EXPOSURE TIME ON MAMMalian CELL SURVIVAL IN VITRO

H. EICHHOLTZ* AND K.-R. TROTT†

From the *Strahlenbiologisches Institut der Universität, Schillerstrasse 42, D-8000 München 2, and the †Institut für Biologie der GSF, D-8042 Neuherberg

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Summary.—Chinese hamster, HeLa and HAK cells were treated with methotrexate (MTX) to determine the dependence of its effect on drug concentration and exposure time. With a broad range of survival curves for Chinese hamster cells, cell survival is an exponential function of exposure time and a power function of drug concentration. The data allow a mathematical description to be made of the interdependence of MTX concentration and drug exposure in relation to cell survival.

Methotrexate (MTX), a folic acid analogue, is a frequently used chemotherapeutic agent in the treatment of various malignant diseases. In clinical schedules, a wide range of doses and overall treatment times has been recommended, ranging from single, high-dose MTX infusions up to 7.5 g/m² (Djerassi et al., 1972; Djerassi, 1975; Jaffe, 1974, and Jaffe et al., 1977; Weichselbaum et al., 1978) to low maintenance doses given over years (Creech et al., 1975, Nagel, 1977). However, the recommended schedules are based on clinical experience rather than on knowledge of the dependence of the cytostatic effect on drug concentration and exposure time.

Survival curves, obtained at varying drug concentrations at one exposure time have been published for HeLa S-3 cells (Berry, 1968) and L-cells (Borsa & Whitmore, 1969). In both cell lines, cell viability decreases with the logarithm of drug concentration until a maximum amount of cell killing is reached at 0.3 μg/ml (surviving fraction 0.1) and 0.02 μg/ml (surviving fraction 0.01) respectively. Bruce et al. (1969) compared the dose and time survival curves for marrow stem cells and lymphoma cells in vivo. With increasing exposure time as with increasing total dose the surviving fraction decreased exponentially to reach a plateau which was different for normal and malignant cells. Pinedo et al. (1977) using constant MTX infusions in mice, related the marrow toxicity to the plasma concentration of MTX, as well as to the duration of drug infusion. They stated that, at constant concentration, the decrease in nucleated cells is an exponential function of exposure time until a plateau is reached.

These studies show that both the exposure time and drug concentration may have a great influence on cell killing by MTX. The interdependence of drug concentration and exposure time, however, has not yet been investigated systematically. The purpose of the present study was to establish the quantitative relationship between drug concentration, exposure time and cell inactivation in vitro.

MATERIAL AND METHODS

Cell lines.—B-14-F-28 Chinese hamster cells, a lung fibroblast cell line, were used (Born, 1974). On subculture they double their cell number within about 24 h and then grow exponentially with a doubling time of 11–14 h. HeLa S-3 and Human adult kidney cells (HAK), both supplied by Flow Labora-
tories, Irvine U.K., were adapted to our culture conditions. Both cell lines have a first doubling time of about 48 h, successive ones being about 24 h.

Cell culture.—All cell lines were cultured in Pyrex bottles using Eagle’s minimum essential medium (Serva, Heidelberg) 10% calf serum (Gibco Bio-Cult, Paisley, U.K.) 0-01% neomycine and 0-035% NaHCO₃.

The stock cultures were trypsinsized (0-25% trypsin for 5 min at 37°C) the cell suspensions diluted to the appropriate cell number and seeded into 4 bottles simultaneously. They were kept in a humidified CO₂ incubator at pH 7·0 and 37°C. One week (Chinese hamster cells) or 2 weeks (HeLa and HAK cells) later, the cells were stained with methylene blue. Colonies consisting of more than 50 cells were counted and the ratio of colony-forming cells of treated cells to controls (the surviving fraction) was calculated.

Drug exposure.—MTX (methotrexate, Lederle) was dissolved in distilled water and kept at 4°C in the dark up to 3 weeks without loss of activity.

HU (hydroxyurea, Boehringer, Mannheim) was dissolved in Hanks’ solution, and kept at −18°C for 3 weeks without loss of activity.

The drugs were added at the time of plating the cells, unless otherwise stated. Exposure time was terminated by removing the medium and carefully rinsing the cells twice with warm Hanks’ solution. Fresh medium was added and the bottles were reincubated for the appropriate time. All experiments were carried out with 4 replicate bottles and repeated at least 3 times. Experimental data were accepted if the colony-forming efficiency of the untreated cells was higher than 35% and if χ² (chi-square) of all replicates was within 95% probability.

Autoradiography.—Chinese hamster cells were plated on microscope slides in Petri dishes. At the end of the exposure to MTX, a final concentration of 0·5 µCi/ml ³H-Thymidine (Amersham, sp. act. 2 Ci/mmole; no known thymidine in the medium) was added to the medium for 30 min at 37°C. After rinsing twice with Hanks’ solution and fixation, the slides were exposed to Eastman Kodak NTB2 emulsion for 2 weeks at 4°C, developed in Kodak D19b and stained with Giemsa solution. 1000 cells were counted to determine the labelling index.

Time-lapse studies.—Chinese hamster cells in T-30 flasks were exposed to 1 µg/ml MTX for 24 h and otherwise treated as above. With a photomicroscope located in an incubator, a picture of the same 10mm² field was taken at 30-min intervals before treatment and for 50–70 h after addition of MTX. The films were analysed according to the method described by Trott (1974), recording for each cell and its progeny the time of cell division and morphological changes like cell pycnosis.

RESULTS

Dependence of the surviving fraction on the concentration of MTX

The surviving fraction of Chinese hamster cells was determined at MTX expo-
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Sure times of 4, 16, 24, 30, and 48 h with drug concentrations increasing from 0.1 to 2.5 μg/ml. Fig. 1 shows the mean of all experimental values with their standard deviations plotted on double-logarithmic scale. The surviving fraction of Chinese hamster cells appears to be a power function of drug concentration.

The cell-killing effect of MTX at one exposure time and varying drug concentrations was also tested for HeLa and HAK cells. Fig. 2 shows the survival curve for HeLa cells at an exposure time of 24 h, plotted on double-logarithmic scale. According to the regression analysis (r² = 0.93) the surviving fraction of HeLa cells is also a power function of drug concentration.

HAK cells were very resistant to the action of MTX. Comparing the concentrations needed to obtain a surviving fraction of 0.1 at an exposure time of 24 h HAK cells displayed a 140-fold lower sensitivity than HeLa cells. They were not considered for further analysis.

**Dependence of the surviving fraction on the exposure time to MTX**

The surviving fraction of Chinese hamster cells was tested for various drug exposure times between 4 and 48 h at MTX concentrations of 0.1, 0.5, 1.0, 2.5 μg/ml. Fig. 3 shows the means of all experimental data with the standard error for each point. Each point represents the mean (+ s.d.) of at least 3 different occasions.

**Fig. 3.—The effect of various exposure times to MTX on the surviving fraction of Chinese hamster cells at constant drug concentration: 0.1 μg/ml (●), 0.5 μg/ml (○), 1 μg/ml (■) and 2.5 μg/ml MTX (□). Each point represents the mean (+ s.d.) of at least 3 different occasions.**

**Fig. 4.—Surviving fraction of HeLa cells to various exposure times to MTX at a drug concentration of 0.1 μg/ml. Each point represents the mean (+ s.d.) of at least 10 dishes, analysed on at least 3 different occasions.**
deviation plotted on semi-logarithmic scale. The exponential curves do not extrapolate back to 100% survival at zero exposure time but have extrapolation numbers > 1. They conform to shoulder curves which are commonly seen after X-irradiation. The extrapolation number decreases from 2·7 at 0·1 μg/ml to 1·3 at 2·5 μg/ml.

Fig. 4 shows the time-dependent survival curve for HeLa cells at a drug concentration of 0·1 μg/ml, which is also exponential. At exposure times > 40 h it may level off.

**DISCUSSION**

The action of MTX depends on both drug concentration and exposure time. However, contrary to suggestions made for cytostatic agents in general (Mellet, 1974) the effect is not simply proportional to the product of concentration and time (i.e. the area under the drug concentration curve). With the above data on the effect of MTX on Chinese hamster cells we want to describe the relationship between MTX concentration and exposure time.

At constant exposure time and drug concentrations varying between 0·1 and 2·5 μg/ml MTX, the surviving fraction decreased according to a power function of concentration, calculated according to the regression analysis in the double-
logarithmic system (0·90 ≤ r² ≤ 0·96):

\[ SF = e^{-at} \cdot bt \]  

(1)

Concentrations higher than 2·5 \( \mu g/ml \) were not tested because the scattering of the results was too high to allow quantitative analysis. Moreover, at this drug concentration, a maximum level of intracellular MTX is achieved in L1210 mouse cells (Goldman et al., 1968) as well as in Yoshida sarcoma cells (Divekar, 1967) due to the saturable influx process. We have no data to suggest a different dependence of the influx upon external drug concentration in our system.

At a given concentration, but with exposure times varying between 16 and 48 h, the surviving fraction decreased according to a shoulder curve with exponential terminal slope (0·93 ≤ r² ≤ 0·98):

\[ SF = e^{-at} \cdot bc \]  

(2)

Due to the presence of a shoulder the results of 4 h exposures were not accounted for in the calculation of the regression lines.

With increasing concentration, the intercept with 100% survival (\( D_0 \), Alper et al., 1962) decreased to lower exposure times from 16 h at 0·1 \( \mu g/ml \) to 2 h at 2·5 \( \mu g/ml \). Furthermore, with increasing concentration the slopes of the time-survival curves increased proportional to the logarithm of the concentration.

Relating both concentration and exposure time to the surviving fraction, assuming that \( at \) is proportional to \( t \) and \( ac \) is proportional to \( \ln \ c \), the following equation was found to be the best simple equation to describe the above data of Chinese hamster cells:

\[ SF (c,t) = k_1 \cdot e^{-k_2t} \cdot c^{-k_3-k_4 \cdot t} \]  

(3)

This equation is identical with both equations (1) and (2).

Keeping \( a \) constant,

\[ a_1 = k_3 + k_4t \]
\[ b_1 = k_1 \cdot e^{-k_2 \cdot t} \]

or keeping \( c \) constant,

\[ a_2 = k_2 + k_4 \ln c \]
\[ b_2 = k_1 \cdot c^{-k_3} \]

With the experimental data (surviving fractions between 0·5 and 0·05 were weighted twice) the general equation can be arranged to:

\[ SF (c,t) = 1.5 \cdot e^{-0.1t} \cdot c^{-0.15-0.02t} \]  

(4)

This explanation of all equations:

\( SF \) = surviving fraction of cells; \( c \) = MTX concentration in the medium in \( \mu g/ml \); \( t \) = duration of exposure in h; \( at \) = slope of the regression line in a double-logarithmic plot, dependent on the exposure time; \( b_1 \cdot e^{\beta} \) (\( \beta \) = intersection of the power function with the ordinate at \( c = 1 \ \mu g/ml \) MTX); \( e \) = base of the natural logarithm; \( ac \) = slope of the regression line in a semi-logarithmic plot, dependent on drug concentration; \( b_c \cdot e^{\beta} \) (\( \beta \) = calculated surviving fraction at the intersection of the exponential survival curve with the ordinate); \( k_1 \cdot 4 \) = varying constants of the general equation.

![Graph](image-url)

**Fig. 6.—** Surviving fraction of Chinese hamster cells to various MTX concentrations: calculated curve from equation (4) superimposed on the experimental data points of Fig. 1.
In inactivation, doubling the concentration from 0.5 μg/ml to 1 μg/ml reduces the exposure time necessary to achieve the same effect from 32.4 to 28.6 h (by a factor of 1.1). Doubling the exposure time, however, from 24 to 48 h reduces the concentration necessary to obtain the same decrease in the surviving fraction by a factor of 8. These results suggest that exposure time is the dominant factor in MTX treatment.

The results of concentration and time dependence of Chinese hamster cells accord with data of Pinedo et al. (1977) who studied mouse marrow toxicity during constant MTX infusion. According to their published results, to get a surviving fraction of 0.4 the concentration of MTX can be reduced by a factor of 25 if the exposure time is increased from 24 to 48 h.

With short exposures, the relative effect of exposure to MTX at low concentration decreases according to what is usually described as the shoulder of a survival curve. Whereas in radiobiology the shoulder is commonly associated with accumulation and repair of sublethal damage, metabolic effects leading to a quasithreshold survival curve are more likely for MTX. So far, however, the concentration-dependent shoulder or threshold of the survival curve cannot be explained by any specific biochemical mechanisms.

In HeLa cells, our results were qualitatively similar but quantitatively different. The surviving fraction decreases according to a power function of concentration and an exponential function of time as it did in Chinese hamster cells.

However it may level off at longer exposures. Since the survival curves of HeLa cells were studied at one concentration or one exposure time only, we cannot calculate a concentration and time dependence for the broad range of concentrations and times as has been done for Chinese hamster cells. However, several points of interest appear: (1) HeLa cells are more sensitive to the action of MTX than Chinese hamster cells; (2) at about
equivalent concentrations determined from Figs 1 and 2 (Chinese hamster cells 0.5 µg/ml; HeLa cells: 0.1 µg/ml) the slope of the regression line for HeLa cells is less steep with increasing exposure time (Chinese hamster cells: \( a_t = -0.093 \), HeLa cells: \( a_t = -0.055 \)). This suggests that the time exponent may be dependent on the growth rate of the cells.

HAK cells were rather resistant to MTX. With an exposure time of 24 h, a drug concentration of about 140 times greater was required to achieve the same level of cell killing as in HeLa cells. The growth rate, and thus the rate of synthesis of new folic-acid reductase (Hakala, 1965) is unlikely to determine the concentration dependence of the action of MTX, since the doubling times of HeLa and HAK cells are identical. The lower sensitivity of HAK than HeLa cells may be explained either by differences in MTX transport into the cells or by different folate-reductase pools (Divekar, 1967) or by the dissociation constants of the dihydrofolate-reductase–MTX complex (Jackson et al., 1976) which, as yet, have not been determined.

The \(^{3}H\)Tdr, HU and time-lapse studies were designed to explore the processes during the prolonged exposure times leading to the loss of unlimited proliferative capacity in Chinese hamster cells. The \(^{3}H\)Tdr labelling of Chinese hamster cells at various times during a 24h MTX treatment showed accumulation of most cells in the S-phase of the cell cycle between 14 and 24 h. Hydroxyurea, however, which selectively kills all cells in S-phase (Sinclair, 1965) had no further statistically significant cell-killing effect after 24 h exposure to MTX.

Most of the cells which continued to incorporate \(^{3}H\)Tdr were already sterilized, since only 10–15% of all cells (the minority of which were in S-phase) were able to form colonies. More information on the mechanism leading to cell inactivation comes from the time-lapse studies. We observed normal cell division continuing for up to 5 h after the start of MTX treatment, probably involving mostly cells already in G2 at the start of MTX exposure. After that time, only 10% of the cells entered mitosis during the following 20h exposure to MTX. During that time most of the cells have been retained in the S-phase as demonstrated in the \(^{3}H\)Tdr experiment. 40% of the cells divided 8–14 h after MTX-free medium was provided. However, most of these dividing cells were not clonogenic but suffered from delayed cell death.

These results fail to confirm the findings of Borsa & Whitmore (1969) who studied the cell-killing action of MTX on L-cells. These authors suggest that MTX at a concentration of 1 µg/ml exposed for 72 h induces antagonistic effects by simultaneous inhibition of DNA, RNA, and protein synthesis, thereby reducing its own killing efficiency. From our findings, there is no indication that Chinese hamster cells are prevented from progressing into the MTX-sensitive S-phase for at least 48 h, since there is an exponential decrease of the surviving fraction with increasing exposure time, to surviving fractions much smaller than the labelling index.

Clinical experience accords with our findings of the importance of exposure time on the cytotoxic effect of MTX, since the marrow toxicity seems to be dramatically enhanced if the serum half life of MTX is prolonged (Sauer & Wilmanns, 1978).

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REFERENCES

Alper, T., Fowler, J. F., Morgan, R. L., Vonberg, D. D., Ellis, F. & Oliver, R. (1962) The characterisation of the "type C" survival curve. Br. J. Radiol., 35, 722.

Berry, R. J. (1968) Some observations on the combined effects of X-rays and MTX on human tumour cells in vitro with possible relevance to their most useful combination in radiotherapy. Am. J. Roentgenol., 102, 509.

Born, R. (1974) Zellkinetische Untersuchungen an chronisch hypoxischen Fibroblasten des Chinesischen Hamsters. Dissertation, Biologische Fakultät der Univ. München.
Borsa, J. & Whitmore, G. G. (1969) Cell killing studies on the mode of action of MTX on L-cells in vitro. Cancer Res., 29, 737.

Bruce, W. R., Meeker, B. E., Powers, W. E. & Valeriote, F. A. (1969) Comparison of the dose- and time-survival curves for normal hematopoietic and lymphoma colony-forming cells exposed to vinblastine, vincristine, arabinosylcytosine and amethopterin. J. Natl Cancer Inst., 42, 1015.

Creeth, R. H., Catalano, R. B., Mastrangelo, M. J. & Engstrom, P. F. (1975) An effective low-dose intermittent cyclophosphamide, methotrexate, and 5-fluorouracil treatment regimen for metastatic breast cancer. Cancer, 35, 1101.

Divekar, A. Y., Vaidya, N. R. & Branganca, B. M. (1967) Active transport of aminopterin in Yoshida sarcoma cells. Biochem. Biophys. Acta, 135, 927.

Djerassi, I., Rominger, C. J., Kim, J. S., Turchi, J., Suvansri, U. & Hughes, D. (1972) Phase I study of high doses of methotrexate with citrovorum factor in patients with lung cancer. Cancer, 30, 22.

Djerassi, I. (1975) High-dose methotrexate (NSC-740) and citrovorum factor (NSC-3590) rescue: background and rationale. Cancer Chemother. Rep., 6, 3.

Goldman, D., Lichtenstein, N. S. & Oliverio, V. T. (1968) Carrier-mediated transport of the folic acid analogue, methotrexate, in the L 1210 Leukemia cell. J. Biol. Chem., 243, 5007.

Hakala, M. T. (1968) On the nature of permeability of Sarcoma-180 cells to amethopterin in vitro. Biochem. Biophys. Acta, 102, 210.

Jackson, R. C., Hart, L. I. & Harrap, K. R. (1976) Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductase. Cancer Res., 36, 1991.

Jaffe, N. (1974) Progress report on high-dose methotrexate (NSC-740) with citrovorum rescue in the treatment of metastatic bone tumors. Cancer Chemother. Rep., 58, 275.

Jaffe, N., Frei, E., Traggis, D. & Watts, H. (1977) Weekly high-dose methotrexate-citrovorum factor in osteogenic sarcoma. Cancer, 39, 45.

Mellert, L. B. (1974) The constancy of the product of concentration and time. In Antineoplastic and Immunosuppressive Agents, Handb. Exp. Pharm. Ed. Sartorelli and Johns. Vol. 38. Berlin: Springer Verlag. p. 203.

Nagel, G. A. (1977) Neue Möglichkeiten der Chemotherapie. Behandlung des metastasierenden Mammacarcinoms. Round-Table-Gespräch: Solide Tumoren. Freiburg: Herausgeber Farmitalia.

Pinedo, H. M., Zaharko, D. S., Bull, J. & Charner, B. A. (1977) The relative contribution of drug concentration and duration of exposure to mouse bone marrow toxicity during continuous MTX infusion. Cancer Res., 37, 445.

Sauer, H. & Wilmanns, W. (1978) Adjuvante Chemotherapie des osteogenen Sarkoms mit High-Dose Methotrexat/Leukovorin: Biochemische Effekte auf die DNA-Synthese der Knochenmarkzellen. Blut, 36, 357.

Sinclair, W. K. (1965) Hydroxyurea: differential lethal effects on cultured mammalian cells during the cell cycle. Science, 150, 1729.

Trott, K. R. (1974) Das Proliferationsmuster unbestrahlter und röntgenbestrahlter Säugetierzellen in vitro (Zeitraffermikrokinematographische Studien). Habilitationsschrift, Medizinische Fakultät der Univ. München.

Weichselbaum, R. R., Miller, D., Pitman, S. W. & Kirkwood, J. (1978) Initial adjuvant weekly high-dose methotrexate with leucovorin rescue in advanced squamous carcinoma of the head and neck. Radiat. Oncol. Biol. Phys. Int. J., 4, 671.