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THE EFFECT OF THE RATE OF CELL PROLIFERATION ON THE SYNTHESIS OF METHOTREXATE POLY-γ-GLUTAMATES IN TWO HUMAN BREAST CANCER CELL LINES

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Abstract—The synthesis of methotrexate poly-γ-glutamates by the MDA-MB-436 and MCF-7 human breast cancer cell lines is highly dependent on the rate of cell growth. Slowly proliferating cells accumulate methotrexate to the same extent as rapidly proliferating cells but convert a lower percentage of the drug to polyglutamate forms. The longest polyglutamate-derivatives of methotrexate are generally only synthesized when the cells are doubling rapidly. The MDA-MB-436 cells exhibit a biphasic response of doubling time and polyglutamation to increasing initial cell number. Extremes of cell density are associated with long doubling times and reduced polyglutamate synthesis. MCF-7 cells show increasing doubling time and decreasing polyglutamate synthesis in response to increasing initial cell number.

The intracellular formation of highly anionic poly-γ-glutamyl derivatives of methotrexate (MTX) was first reported by Baugh *et al.* [1]. Since then, the formation of MTX polyglutamates has been reported in a wide variety of tissues including breast cancer [2–8]. Methotrexate polyglutamates can bind to dihydrofolate reductase (DHFR) [9, 10] and are at least as effective as the parent drug in causing inhibition of that enzyme [11]. Furthermore, MTX polyglutamates persist in human tissue for long periods after cessation of treatment with MTX [12]. The retention of MTX polyglutamates by human breast cancer cells *in vitro* is proportional to the length of the γ-glutamyl chain [5]. Thus the formation of γ-glutamyl derivatives of MTX by cells may be a major factor in determining the therapeutic and toxic response to the drug.

However, comparatively little is known about the mechanism by which the synthesis of MTX polyglutamates is regulated. Biosynthesis of these metabolites may be hormonally regulated, since increased MTX polyglutamate synthesis was observed when human breast cancer cells or rat hepatoma cells were cultured in the presence of insulin [5, 13, 14]. Furthermore, dexamethasone can stimulate MTX polyglutamate synthesis [14]. Methotrexate polyglutamylation may also be regulated by the folate status of the cell, since folate-deficient cells synthesize higher levels of MTX polyglutamates than folate-sufficient cells [13, 15]. Vincristine and the epipodophyllotoxins, VM-26 and VP-16-213, may also increase polyglutamate synthesis [16, 17]. These compounds increase the free intracellular drug level by inhibiting MTX efflux from the cells, thus providing a higher substrate concentration for the enzyme folylpolyglutamate synthetase.

On the other hand, Actinomycin-D, cycloheximide, dibutyryl cAMP and theophylline [14] and leucovorin [8, 18] can decrease the synthesis of MTX polyglutamates.

In the present study we have examined the influence of initial cell-plating density on the rate of cell proliferation and MTX polyglutamate synthesis in the MCF-7 and MDA-MB-436 human breast cancer cell lines.

MATERIALS AND METHODS

3′,5′,7-[^{3}H]Methotrexate (TRK 224; sp. act. 11.9 Ci/mmole) was purchased from Amersham International (Amersham, U.K.). The purity of the label was not less than 98%, as determined by paper chromatography using *n*-butanol:pyridine:water (1:1:1) as the eluent system. Methotrexate polyglutamate standards were purchased from Dr. C. M. Baugh (University of South Alabama, Mobile, AL, U.S.A.). The MCF-7 cells were a gift from Dr. C. D. Green (University of Liverpool, U.K.) and were originated by Soule *et al.* [19]. The MDA-MB-436 cell line, the characteristics of which have previously been described [20], were obtained from Flow Laboratories Ltd. (Irvine, Scotland). Foetal calf serum was supplied by Randox Laboratories (Crumlin, U.K.) and was dialysed extensively against physiological saline before use.

Cell culture conditions. Cells (10^4–10^6) were plated onto petri dishes (20 cm^2 surface area; Sterlin Ltd, Teddington, U.K.) in Eagles Minimal Essential Medium supplemented with Earles salts, 5% dialysed serum, penicillin (100 I.U./ml) and streptomycin (100 μg/ml). The cells were grown for 72 hr in an air:CO_2 (95:5) atmosphere at 37° and were then exposed to fresh medium containing 10^{-7} M [^{3}H]-MTX. Following exposure of the cells to [^{3}H]MTX for 48 hr the cell monolayer was washed twice with ice-cold isotonic phosphate-buffered saline pH 7.4

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(3.0 ml) and once with ice-cold 0.15 M sodium phosphate, pH 7.4 (3.0 ml). The measurement of intracellular drug levels, dihydrofolate reductase levels and extraction of MTX polyglutamates from the cell monolayers was accomplished as previously described [8]. Cell numbers were determined electronically using a Coulter Counter 24 and 48 hr after plating out the cells. Doubling times were calculated using the formula:

$$\text{Doubling time} = T_{\text{final}} - T_{\text{init}}/3.32(\log \text{cell no.}\ T_{\text{final}} - \log \text{cell no.}\ T_{\text{init}}).$$

High pressure liquid chromatography. Methotrexate and methotrexate polyglutamates were separated using a paired-ion high pressure liquid chromatography (HPLC) system (Waters Associates). The mobile phase was prepared by mixing the effluents of a model M-45 and a model 6000-A pump under the direction of a model 720 system controller. Samples were injected onto a μ-Bondapak C_{18} reverse-phase column (0.39 × 30 cm) using a model U6K injector. Solvent A consisted of 10 mM potassium dihydrogen orthophosphate pH 7.0 containing 5 mM tetrabutyl ammonium hydrogen sulphate and solvent B consisted of methanol. The column was irrigated at a flow rate of 1.5 ml/min with 30–40% methanol over 25 min and 40% methanol over 12 min. The retention times of MTX and MTX polyglutamates were determined by measuring the absorbance of the effluent at 254 nm using a model 441 detector. Column effluent was collected as 27-drop fractions into disposable scintillation-vial inserts using an LKB 2112 Redirac fraction collector.

Scintillation fluid (4 ml of 0.2% PPO in toluene: Triton X-100 [2:1 (v/v)] was added and radioactivity determined using a Rackbeta 1217 liquid scintillation spectrometer. Recovery of radioactivity from the HPLC column was 92.0 ± 8.9% (Mean ± S.E.M.).

RESULTS

When cell extracts containing MTX polyglutamates were subjected to analysis using HPLC, peaks of radioactivity corresponding to authentic polyglutamate standards (MTX to MTXG5, which contains five additional γ-glutamyl residues) were detected. However, further peaks of radioactivity were detected which eluted from the column after MTXG5. These were provisionally designated MTXG6–MTXG10 on the basis of the relationship established by Cashmore *et al.* [21] between the logarithm of the adjusted retention time (t_r') and the square root of the total number of glutamate residues (Fig. 1 of reference [8]). The correlation coefficient of regression for this relationship (0.997) is comparable with the values of 0.995 and 0.999 which have previously been reported [3, 8].

MDA-MB-436 and MCF-7 cells were plated out at initial cell numbers between 10^4 and 10^6 cells per dish. The intracellular drug levels in the MDA-MB-436 cell line (3.43 ± 0.28 pmoles/mg cell protein) and the MCF-7 cell line (4.39 ± 0.18 pmoles/mg cell protein) were unaffected by differences in plating density ($P > 0.1$, Student's t-test). The DHFR levels (0.91 ± 0.10 and 2.30 ± 0.20 pmoles/mg cell protein) in the MDA-MB-436 and MCF-7 cell lines, respectively, were determined as described in the companion paper [8]. The number and doubling time of the cells immediately before exposure to MTX is shown in Table 1. The doubling time of the MCF-7 cells increased with increasing cell density. However, that of the MDA-MB-436 cell line reached a minimum at cell densities of 5×10^4 and 10^5 cells per plate. To either side of these densities the doubling times increased substantially.

At any particular seeding density the two cell lines differed in their ability to synthesize polyglutamates (Table 2). Furthermore, each cell line exhibited a differential response to initial cell number in respect of their ability to synthesize MTX polyglutamates. The MDA-MB-436 cell line converted 87% of the intracellular drug to polyglutamyl derivatives at initial cell densities of 5×10^4 and 10^5 cells per plate. However, at lower (10^4) and higher (5×10^5 and 10^6) initial cell densities, the proportion of the intracellular drug present as MTX polyglutamates fell to 53 and about 77%, respectively. Furthermore, small amounts of the very high molecular weight species designated MTXG9 and MTXG10, were detected at the highest initial plating densities.

In contrast no biphasic response to cell density was observed in the MCF-7 cell line. The proportion of the intracellular drug present as polyglutamates decreased with increasing numbers of cells initially plated. The synthesis of MTXG7 and MTXG8 was completely abolished at initial cell densities greater than 10^5 cells per plate.

There is a strong correlation between the proportion of the intracellular drug in the form of MTX and the doubling times of the two cell lines (Fig. 1). The MDA-MB-436 cell line exhibited a biphasic response of both doubling time and drug metabolism to plating density. In contrast the MCF-7 cell line showed a direct relationship between plating density

Table 1. Cells (10^4–10^6) were plated onto 20-cm^2 petri dishes and cultured for 72 hr. Cell doubling times (D.T., hr) were calculated from measurements of cell number 24 and 72 hr after seeding

	Cell line			
	MDA-MB-436		MCF-7	
Density	Cell No. (Day 3)	D.T.	Cell No. (Day 3)	D.T.
10^4	6.49×10^3	76.2	2.33×10^4	24.9
5×10^4	7.15×10^4	44.0	1.01×10^5	30.8
10^5	2.66×10^5	29.6	1.58×10^5	37.2
5×10^5	4.05×10^5	96.4	2.81×10^5	154
10^6	6.44×10^5	104.3	3.91×10^5	∞

Table 2. The effect of initial cell number on the percentage distribution of intracellular MTX amongst its poly-γ-glutamyl derivatives. Cells were cultured for 72 hr before being exposed to medium containing 10^{-7} M [^{3}H]MTX. Intracellular formation of MTX polyglutamates was assessed after a 48-hr exposure to the drug. Values represent a mean from two separate experiments and are subject to an average error of less than 7%

Species	Initial cell No.				
	10^4	5×10^4	10^5	5×10^5	10^6
MDA-MB-436					
MTX	47.7	13.0	13.1	22.9	24.7
MTXG1	14.3	10.1	10.3	14.7	14.4
MTXG2	9.7	21.5	24.4	17.5	16.8
MTXG3	14.4	15.7	13.0	9.4	10.4
MTXG4	14.0	17.6	14.4	7.0	3.5
MTXG5		4.8	4.6	7.7	5.8
MTXG6		6.6	3.6	5.8	5.8
MTXG7		9.1	11.7	6.6	4.1
MTXG8		1.8	5.4	2.9	6.7
MTXG9				2.8	5.1
MTXG10				3.0	2.8
MCF-7					
MTX	20.5	29.1	46.4	69.0	72.7
MTXG1	7.3	9.6	10.3	8.6	10.9
MTXG2	11.4	14.6	12.5	10.0	9.4
MTXG3	15.8	13.8	10.4	3.4	2.1
MTXG4	15.8	14.8	7.3	3.6	3.6
MTXG5	8.2	5.6	2.9	1.0	0.8
MTXG6	7.6	2.6	1.7	0.6	0.5
MTXG7	8.1	4.0	2.4		
MTXG8	5.3	4.9	4.2		

and both doubling time and drug metabolism. At 10^6 cells per dish the MCF-7 cells were fully confluent and the cell number did not increase during the course of the experiment.

DISCUSSION

When plated out at low density, MCF-7 and MDA-MB-436 cells synthesize polyglutamates which co-chromatograph with authentic MTXG1–MTXG4 as previously described [3, 4]. However, they also synthesize material which co-chromatographs with MTXG5 and material with the projected chromatographic characteristics of MTXG6–MTXG8 and MTXG6–MTXG10, respectively (Fig. 1 of reference [8] and Table 2). Furthermore, the radioactive species can bind to DHFR as described previously [8].

There is a strong negative correlation between growth rate and the synthesis of MTX polyglutamates in these cell lines (Table 2, Fig. 1). However, a noteworthy difference in their behaviour is the biphasic response of the MDA-MB-436 cells to variations in plating density (Fig. 1). This may be as a result of the secretion into the culture medium of factors which promote cell growth [22]. However, the precise reason for this is unclear. In contrast the doubling time of the MCF-7 cell line increased with increasing cell number within the range studied.

The synthesis of MTX polyglutamates by both cell lines is also strongly dependent on cell density. MDA-MB-436 cells show a biphasic relationship between polyglutamylation and initial cell density (Table 2) similar to that between growth rate and cell density. This cell line has an optimum range of cell densities (5×10^4–10^5 cells per dish) which afford rapid growth and extensive metabolism of the drug to polyglutamates. At lower or higher densities, a larger proportion of the intracellular antifolate is present as the parent drug. In contrast no optimum cell density was apparent for the MCF-7 cell line, within the range studied. Increasing cell density led to both an increase in cell doubling time and a decrease in the percentage contribution of MTX polyglutamates to total intracellular drug. Rosenblatt *et al.* [23] have previously demonstrated decreased synthesis of MTXG1 and MTXG2 as human fibroblasts approached confluence, without an increase in levels of the parent drug. Similarly Nimec *et al.* [13] have reported a decrease in the rate

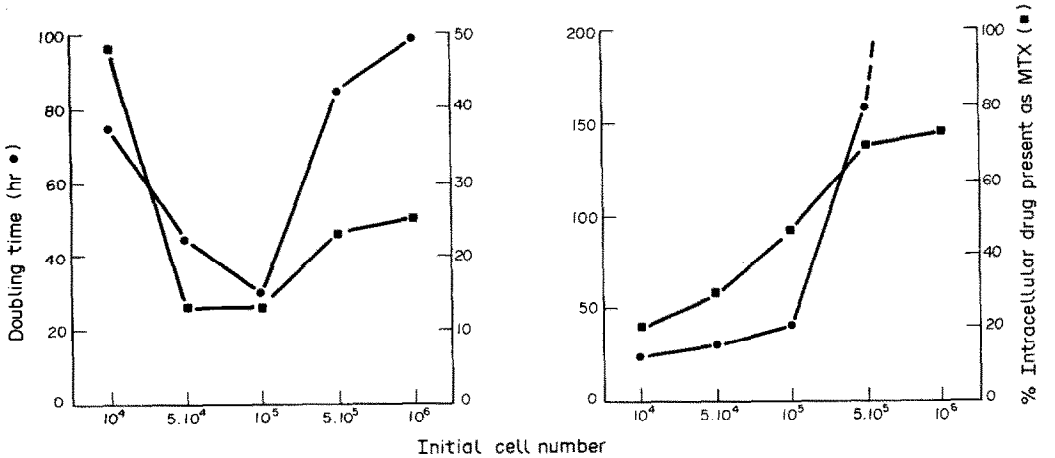

Fig. 1. The effect of initial cell number on the doubling time (●) and percentage of the intracellular drug present as MTX (■) in the MDA-MB-436 and MCF-7 human breast cancer cell lines. Cells were cultured for 72 hr before being exposed to medium containing 10^{-7} M [^{3}H]MTX. MTX polyglutamate formation was assessed after a 48 hr exposure to the drug. Doubling times were measured before exposure to MTX.

of glutamylation of MTX with increasing confluence of rat H-35 hepatoma cells.

The present study may provide an explanation for the finding that MTX polyglutamate species up to MTXG8 could be detected in extracts derived from the MCF-7 human breast cancer cell line ([8] and Table 2). Other workers using this cell line [2–4, 6] have failed to detect any polyglutamates longer than MTXG5. Table 2 shows that the MCF-7 cell line can synthesize polyglutamates with up to 8 additional γ-glutamyl residues, providing that the cells are plated out at low density. Reduced synthesis of polyglutamates was observed at the highest plating densities, with the bulk of the species observed being shorter than MTXG5. Previous workers, however, did use initial cell numbers which may have precluded the detection of the larger molecular weight species reported here. The area of the petri dishes used in the present study was approximately 20 cm^2, giving a maximum plating density of 5×10^4 cells/cm^2. Jolivet *et al.* [3] plated 10^7 cells onto a 75-cm^2 flask, equivalent to 1.3×10^5 cells/cm^2. Our data suggest that at such high plating densities, MTX polyglutamates longer than MTXG4 are unlikely to be detected.

Methotrexate, a phase-specific agent, is most effective against exponentially growing cells as opposed to those in the stationary phase [24, 25]. Furthermore, MTX polyglutamates are at least as good inhibitors of DHFR as MTX itself [11] and their cellular retention increases with increasing γ-glutamyl chain length [5]. Hence the decreased rate of polyglutamylation in slowly proliferating cells reported in the present study may provide an additional mechanism by which slowly proliferating cells are more resistant to MTX.

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REFERENCES

1. C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. biophys. Res. Commun.* **52**, 27 (1973).
2. R. L. Schilsky, B. D. Bailey and B. A. Chabner, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2919 (1980).
3. J. Jolivet and R. L. Schilsky, *Biochem. Pharmac.* **30**, 1387 (1981).
4. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
5. D. G. Kennedy, R. Clarke, H. W. Van den Berg and R. F. Murphy, *Biochem. Pharmac.* **32**, 41 (1983).
6. J. Jolivet and B. A. Chabner, *J. clin. Invest.* **72**, 773 (1983).
7. K. H. Cowan and J. Jolivet, *Clin. Res.* **31**, 508 (1983).
8. D. G. Kennedy, H. W. Van den Berg, R. Clarke and R. F. Murphy, *Biochem. Pharmac.* **34**, 2897 (1985).
9. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
10. M. Chauvet, M. Bordeaux, C. Briand, M. Dell'Amico, R. Gilli and M. Diarra, *Biochem. Pharmac.* **32**, 1059 (1983).
11. S. A. Jacobs, R. H. Adamson, B. A. Chabner, C. J. Derr and D. G. Johns, *Biochem. biophys. Res. Commun.* **63**, 692 (1975).
12. S. A. Jacobs, C. J. Derr and D. G. Johns, *Biochem. Pharmac.* **26**, 2310 (1977).
13. Z. Nimec and J. Galivan, *Archs Biochem. Biophys.* **226**, 671 (1983).
14. J. Galivan, *Archs Biochem. Biophys.* **230**, 355 (1984).
15. J. Galivan, Z. Nimec and M. Balinska, *Biochem. Pharmac.* **32**, 3244 (1983).
16. D. W. Fry, J. C. Yalowich and I. D. Goldman, *Cancer Res.* **42**, 2352 (1982).
17. J. C. Yalowich, D. W. Fry and I. D. Goldman, *Cancer Res.* **42**, 3648 (1982).
18. J. Galivan and Z. Nimec, *Cancer Res.* **43**, 551 (1983).
19. H. D. Soule, J. Vazquez, A. Long, S. Albert and M. Brenna, *J. natn. Cancer Ints.* **51**, 1409 (1973).
20. R. Clarke, H. W. Van den Berg, D. G. Kennedy and R. F. Murphy, *Eur. J. Cancer clin. Oncol.* **19**, 19 (1983).
21. A. R. Cashmore, R. N. Dreyer, C. Horvath, J. O. Knipe, J. K. Coward and J. R. Bertino, *Meth. Enzymol.* **66**, 459 (1980).
22. F. Vignon, D. Derocq, M. Chambon and H. Rochefort; *C.r. hebd. séanc. Acad. Sci., Paris,* **296**, 151 (1983).
23. D. S. Rosenblatt, V. M. Whitehead, M. M. Dupont, M. J. Vuchich and N. Vera, *Molec. Pharmac.* **14**, 210 (1978).
24. J. Borsa and G. F. Whitmore, *Cancer Res.* **29**, 737 (1969).
25. W. M. Hryniuk, G. A. Fischer and J. R. Bertino, *Molec. Pharmac.* **5**, 557 (1969).