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THE KINETICS OF METHOTREXATE POLYGLUTAMATE FORMATION AND EFFLUX IN A HUMAN BREAST CANCER CELL LINE (MDA.MB.436): THE EFFECT OF INSULIN

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Abstract—The rate and extent of the formation of methotrexate poly-γ-glutamates has been studied in a human breast cancer cell line (MDA.MB.436). Cells were exposed to medium containing 10^{-7} M radiolabelled methotrexate (MTX) for various periods. Cell extracts were subjected to gel filtration on Bio-Gel P2 and radioactivity in each fraction determined. This has allowed quantitation of the relative amounts of MTX and its polyglutamate derivatives. It has been proposed that MTX polyglutamates may act as storage forms of the drug which could result in prolonged cytotoxicity. This possibility was investigated by determining the ability of the cell line to retain MTX and its polyglutamate derivatives for periods of up to 48 hr after removal of MTX from the incubation medium. Our results show that the lower mol. wt polyglutamates (< three extra glutamic acid residues) rapidly efflux from the cell, while the higher mol. wt species (> three glutamic acid residues) are extensively retained, having an efflux half-life of approximately 30 hr. It has been shown that insulin may potentiate the cytotoxicity of MTX to breast cancer cells *in vitro*. Our results indicate that in the MDA.MB.436 cell line the total intracellular drug at the steady state is unaffected by insulin (10^{-6} M). However insulin does result in a 30% increase in the contribution of the higher mol. wt polyglutamates to the total intracellular drug. Our data therefore suggest that the ability of insulin to potentiate the cytotoxic effects of MTX may be related to the hormone's ability to modulate the synthesis of MTX polyglutamates.

The formation of poly-γ-glutamyl derivatives of folic acid was first reported in 1946 [1]. Since then it has been well established that most intracellular folates can be linked to as many as seven additional γ-glutamyl residues. The first report in the literature on the formation of polyglutamate derivatives of the dihydrofolate reductase inhibitor, methotrexate (MTX), was in 1973 [2]. In this study MTXG1 and MTXG2 (containing one and two γ-glutamyl residues respectively) were isolated from rat liver and detected in human red blood cells. More recently MTX polyglutamate synthesis has been observed in a variety of normal and neoplastic cell lines [3–6] including those derived from human breast cancer [7, 8]. However the rate and extent of polyglutamate formation varies widely [3, 4] and it has been proposed that this may in part account for observed differences in sensitivity towards the drug. However, Poser *et al.* [5] demonstrated that differential polyglutamate synthesis could not account for differences in sensitivity of murine tissues to MTX. Similarly there is considerable disagreement in the literature regarding the persistence of polyglutamate derivatives within the cell. Some workers [9] have shown that all of the polyglutamate forms and MTX itself efflux from the cell with similar facility, whereas others [10, 11] suggest that the polyglutamates may be selectively retained. Polyglutamates have been shown to be at least as active as MTX as inhibitors of dihydrofolate reductase [7, 11, 12] but their precise pharmacological role remains uncertain.

It has been demonstrated that insulin can enhance the cytotoxicity of MTX in a human breast cancer cell line (MCF-7) [13]. The precise mechanism by which insulin exerts this effect remains unknown although the potentiation was not associated with an alteration in steady-state intracellular MTX levels [13]; however drug transport kinetics have been reported to be affected by insulin [14].

In the present study we have examined the influx of MTX and its conversion to polyglutamates by a human breast cancer cell line (MDA.MB.436). In addition we have assessed the ability of these cells to retain MTX and its polyglutamate derivatives after exposure to the parent drug. The influence of insulin on these parameters has also been studied in an attempt to cast light on the mechanism of insulin-induced potentiation of MTX action in human breast cancer cells [13].

MATERIALS AND METHODS

[3′,5′,7′-^{3}H]Methotrexate (TRK224; sp. act. 20 Ci/mmole) was purchased from Amersham International Ltd (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 (v/v/v)] as the eluent system. MDA.MB.436 human breast cancer cells were obtained from Flow Laboratories Ltd (Irvine, U.K.). Petri dishes (5-cm diameter) were purchased from Sterilin Ltd (Teddington, U.K.). Bovine pancreatic

insulin and bovine liver dihydrofolate reductase were obtained from Sigma Chemical Co. (Poole, U.K.). Foetal calf serum was obtained from Gibco Europe Ltd (Paisley, U.K.) and was rendered free of endogenous hormones by the use of activated charcoal. Human serum albumin was obtained from Behringwerke AG (Mannheim, F.R.G.).

Cell culture conditions. The MDA.MB.436 cell line was derived from a pleural effusion of a stage 3 breast carcinoma [15] and possesses features characteristic of human breast epithelia [15, 16].

Approximately 5×10^5 cells were plated into 5-cm petri dishes in Eagle's minimal essential medium supplemented with Earle's salts, 5% charcoal-stripped foetal calf serum, 100 I.U./ml penicillin, 100 μg/ml streptomycin with and without 10^{-6} M insulin. The cells were grown in an air:CO_2 atmosphere [95:5 (v/v)] at 37°. After 3–4 days the medium was replaced with medium containing 10^{-7} M [^{3}H]MTX with and without insulin. Influx of MTX and polyglutamate formation was assessed at various times up to 48 hr. Quadruplicate dishes were used at each time point, three for assessment of total intracellular radioactivity and one to estimate the formation of MTX polyglutamates. At each time point the medium containing the tritiated drug was removed and the monolayer washed 4 times with 3-ml aliquots of ice-cold isotonic phosphate-buffered saline, pH 7.4 (PBS). Efflux of the drug from the cells and the polyglutamate distribution of the retained drug was measured by preloading the cells for 48 hr with medium containing 10^{-7} M [^{3}H]MTX with and without 10^{-6} M insulin. The monolayer was washed 4 times with sterile, ice-cold PBS and 3 ml fresh drug-free medium with and without insulin was added. Efflux of the drug from the cells was followed for periods of up to 48 hr as described earlier.

Measurement of total intracellular radioactivity. The cell monolayer was dissolved in 1.0 ml 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.001 M EDTA, 0.5% (w/v) sodium dodecyl sulphate and 0.02% (w/v) sodium azide. Five hundred microlitres of the resulting solution was removed and radioactivity determined using an Intertechnique SL-30 liquid scintillation spectrometer with a counting efficiency of 49%. The remainder of the solution was assayed for protein content [17] using human serum albumin to prepare the standard solutions. The results were corrected for counting efficiency, protein content and the sp. act. of the label and were expressed as fmoles MTX/mg protein.

Estimation of poly-γ-glutamate formation. The cell monolayer was suspended in 2.0 ml PBS using a rubber policeman and transferred to a tapered centrifuge tube which was placed in a boiling water bath for 10 min to disrupt the cells and inactivate intracellular peptidases. The cell debris was pelleted by centrifugation at 5000 *g* for 15 min and the supernatant stored at −20° until required.

The cell extracts were subjected to gel filtration on a 0.9×80 cm column of Bio-Gel P2 which had previously been equilibrated at 4° with 20 mM ammonium bicarbonate buffer, pH 8.0, containing 0.02% (w/v) sodium azide. The column was irrigated with the same buffer at a flow rate of 20 min/ml and 1.0-ml fractions were collected. Radioactivity in each fraction was determined and the proportion of the total intracellular radioactivity contributed by each of the polyglutamate species estimated by calculating the area under the curve for each of the peaks on the elution profile.

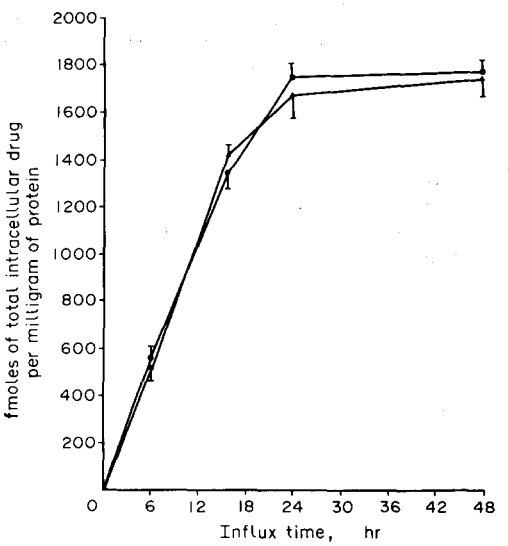

Fig. 1. Rate of influx of total drug following exposure of MDA.MB.436 cells to 10^{-7} M [^{3}H]MTX (●——●) or 10^{-7} [^{3}H]MTX in the presence of 10^{-6} M insulin (▲——▲). Mean ± S.D. of three determinations.

Assessment of polyglutamate distribution of effluxed drug. The medium into which the intracellular drug was allowed to efflux was analysed for polyglutamate content as described earlier with the exception that the medium was supplemented with 50 μg of bovine dihydrofolate reductase to trap extracellular polyglutamates so protecting them from hydrolysis by extracellular peptidases [7]. The amounts of each of the species apparently lost from the cells during a 2-hr efflux period were compared with the recovery of those species in the medium.

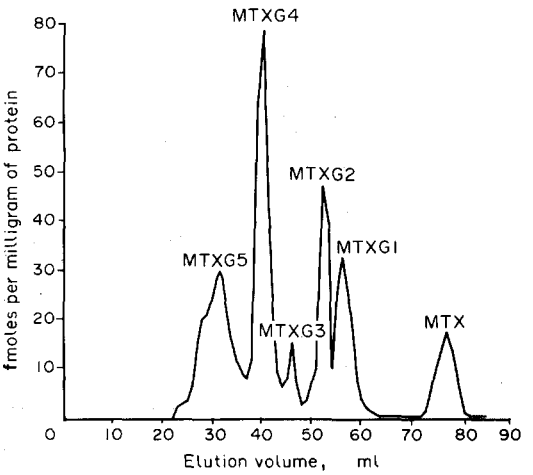

Fig. 2. Elution profile of MTX and polyglutamates subjected to gel filtration on a 0.9×80 cm column of Bio-Gel P2.

Table 1. The distribution of MTX and polyglutamates expressed as a percentage of total intracellular drug after various periods of exposure to 10^{-7} M [^{3}H]MTX with (+) and without (−) 10^{-6} M insulin

	6°		16°		24°		48°	
Species	−	+	−	+	−	+	−	+
MTX	21.0	41.5	12.1	18.1	11.7	13.5	17.2	11.3
MTXG1	31.5	17.3	23.4	12.9	20.7	9.7	10.5	7.3
MTXG2	12.8	11.8	14.6	11.5	15.0	2.5	10.3	2.0
MTXG3	8.6	8.6	5.7	5.7	5.6	9.4	6.0	8.4
MTXG4	19.5	15.0	27.1	28.6	26.6	39.4	26.3	31.2
MTXG5	6.5	3.6	17.0	23.0	20.3	24.9	29.6	39.7

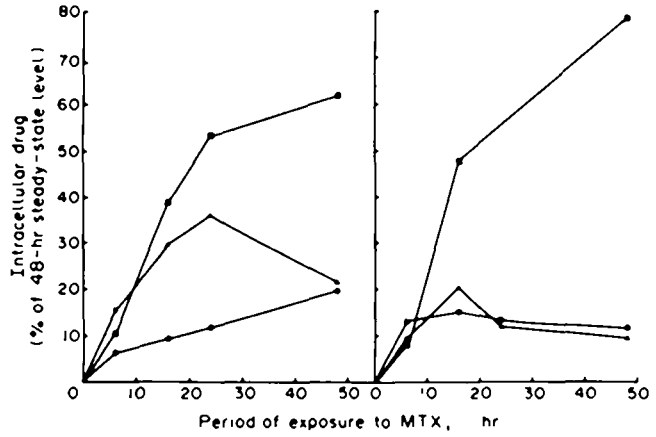

Fig. 3. Accumulation of intracellular MTX and polyglutamates following exposure of MDA.MB.436 cells to 10^{-7} M [^{3}H]MTX in the absence (left panel) and presence (right panel) of 10^{-6} M insulin. Key: (●——●) MTX; (▲——▲) MTXG1 + MTXG2; (■——■) MTXG3–MTXG5.

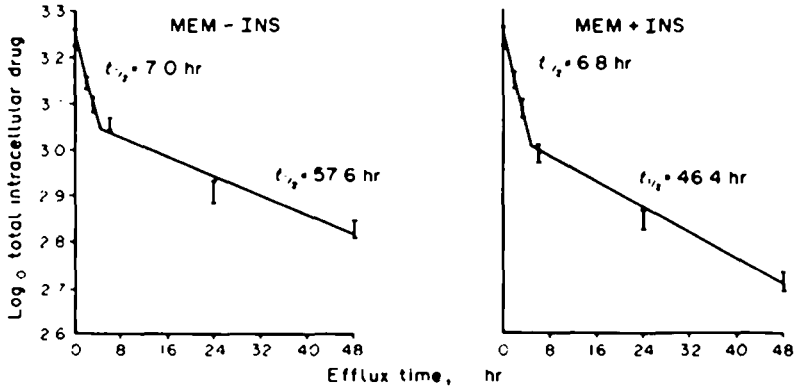

Fig. 4. Rate of total drug efflux from MDA.MB.436 cells following a 48-hr period of exposure to 10^{-7} M [^{3}H]MTX in the absence or presence of 10^{-6} M insulin. Mean ± S.D. of three determinations.

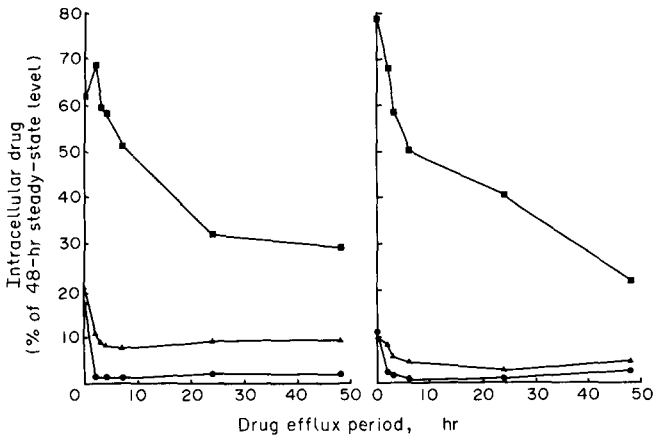

Fig. 5. Rate of efflux of MTX and polyglutamates from MDA.MB.436 cells following a 48-hr period of exposure to 10^{-7} M [^{3}H]MTX in the absence (left panel) or presence (right panel) of 10^{-6} M insulin. Key: (●——●) MTX; (▲——▲) MTXG1 + MTXG2; (■——■) MTXG3–MTXG5.

Table 2. Initial half-lives of efflux of MTX and polyglutamates following a 48-hr exposure of cells to 10^{-7} M [^{3}H]MTX with and without 10^{-6} M insulin

Species	$t_{\frac{1}{2}}$ (hr) − insulin	$t_{\frac{1}{2}}$ (hr) + insulin
MTX	0.59	1.57 ± 0.3
MTXG1	4.30 ± 0.9	4.3 ± 0.5
MTXG2	1.4 ± 0.1	5.7 ± 0.9
MTXG3	20.5 ± 5.2	17.5 ± 2.2
MTXG4	27.2 ± 4.1	26.1 ± 3.6
MTXG5	33.5 ± 9.8	31.5 ± 4.0

RESULTS

Fig. 1 shows that insulin failed to alter either the initial rate of influx or the steady-state intracellular levels of MTX. Fig. 2 shows a typical elution profile of MTX and polyglutamates obtained using Bio-Gel P2. The void volume of the column, as judged by the elution of haemoglobin, was 21 ml and non-radioactive MTX eluted at 76 ml. The mean recovery of radioactivity from the column was 91.6 ± 6.9% (S.E.M.) (P = 0.05). The other radioactive compounds eluting from the column were designated (in order of increasing mol. wt) MTXG1–MTXG5. We have been unable to obtain authentic samples of any of these species to confirm their identity, but there is no evidence that these metabolites elute from gel filtration systems in any order other than that of descending mol. wt. The effect of insulin on the contribution to the total intracellular drug of each of the polyglutamate derivatives during a 48-hr exposure period is shown in Table 1. Insulin decreased the time at which the lower mol. wt species (G < 3) reached their maximum level before declining, whereas the higher mol. wt species (G > 3) either reached a steady state or continued rising in both the presence and absence of insulin. The identity of the single predominant species between 16 and 48 hr was unaffected by insulin being MTXG4 at 16 and 24 hr and MTXG5 at 48 hr. Fig. 3 displays these results graphically where several of the species have been grouped together on the basis of their behaviour during influx. This shows that there was a 30%

Table 3. Amounts of MTX and polyglutamates lost during a 2-hr efflux period from cells preloaded with 10^{-7} M [^{3}H]MTX for 48 hr compared to amounts recovered in the growth medium

Species	Species lost from cells (fmoles/mg cellular protein)	Species recovered in medium (fmoles/mg cellular protein)
MTX	152	137
MTXG1	30	73
MTXG2	14	47
MTXG3	59	32
MTXG4	84	37
MTXG5	28	0
Total	367	326

The amount of each species lost from the cells was calculated from the difference between species distribution at the end of the 48-hr incubation period (Table 1) and the distribution following a 2-hr efflux period (Fig. 5). Species distribution in the growth medium was determined following gel filtration of the medium as described in the text.

increase in the contribution to total intracellular drug of the higher mol. wt species (MTXG3—MTXG5), in the presence of insulin. Total drug efflux from the MDA.MB.436 cell line, shown in Fig. 4, was a biexponential process. The first, rapid phase of efflux has a half-life of about 7 hr and the second, slower phase of efflux a half-life of about 58 hr. these half-lives were not significantly altered in the presence of insulin. The retention by the cells of MTX and its polyglutamate forms is shown in Fig. 5. Although MTX and the lower mol. wt species initially effluxed rapidly from the cells a steady state was reached within about 8 hr. Initial half-lives of efflux for MTX and each of the polyglutamates were calculated and are displayed in Table 2. The rates of efflux were not significantly affected by insulin; however our data clearly demonstrate that efflux half-lives are proportional to the γ-glutamyl chain length. Furthermore this relationship does not appear to be a direct one since we observed a disproportionate increase in efflux half-life between MTXG2 and MTXG3.

Table 3 compares the apparent loss of drug from the cells and the drug recovered in the incubation medium following a 2-hr efflux period. MTX and polyglutamate loss from the cells was calculated from the data shown in Table 1 and Fig. 5. It will be observed that 28 fmoles/mg cellular protein MTXG5 was apparently lost from the cells during the efflux period whilst this species did not appear in the culture medium. In addition, considerably less MTXG3 + MTXG4 was recovered in the medium than was apparently lost from the cells. This suggests that intracellular loss of MTXG5 and to a lesser extent MTXG3 and MTXG4 is the result of conversion to lower mol. wt species. This is supported by the finding that more than $2\frac{1}{2}$ times the amount of MTXG1 and MTXG2 lost from the cell was recovered in the medium.

DISCUSSION

The ability of insulin to potentiate the cytotoxicity of MTX towards human breast cancer cells does not appear to result from alterations in influx, steady-state levels or efflux of the drug (Figs. 1 and 4). This is an agreement with an earlier report [13] which showed that insulin did not modify the steady-state intracellular levels achieved in a human breast cancer cell line (MCF-7). However the time taken to reach the steady state reported here (24 hr with 10^{-7} M MTX) is considerably longer than that reported in the earlier study (2 hr with 5×10^{-8} M MTX).

Extensive conversion of MTX to its polyglutamate derivatives occurs in the MDA.MB.436 cell line. After 48 hr incubation with MTX about 85% of the total intracellular drug is in the form of MTX polyglutamates (Table 1). Furthermore, insulin modifies the synthesis of MTX polyglutamates resulting in a 30% increase in the contribution of the higher mol. wt species to total intracellular drug from about 16 hr onward. This increase is mainly at the expense of the lower mol. wt species rather than the parent drug (Fig. 3). Fig. 4 shows that insulin does not affect the half-lives of efflux of total drug from the cell. Since this result would not be inconsistent with insulin-induced changes in efflux of individual polyglutamate forms of MTX, these were calculated as shown in Table 2. The half-lives of efflux were not significantly affected by the presence of insulin; however they are proportional to the γ-glutamyl chain length. The relationship is not linear, there being a disproportionate increase in half-life between MTXG2 and MTXG3. This suggests that the higher mol. wt species may have a distinct role to play in the pharmacology of the drug, possibly as storage forms. The absence of recovery in the medium of any MTXG5 or of substantial amounts of MTXG3 and MTXG4 suggests that these species may first undergo intracellular hydrolysis before effluxing from the cell. Although amounts of MTXG3 and MTXG4 were detected in the medium they did not exceed 50% of the total amount of those species that was apparently lost from the cells. (Table 3). This taken together with the data shown on Table 2 tends to confirm that MTXG3, MTXG4 and MTXG5 are storage forms of drug. This in contrast with a previous study [9] in which it was reported that L1210 cells exhibited no retentive capacity for the polyglutamates. It has been shown [18] that human fibroblast cells in culture do not have an inherent ability to retain polyglutamates, but that their disposition results in their gradually acquiring an ability to be retained within the cell by forming a "non-exchangeable pool". This earlier report demonstrated that the ability of the polyglutamates to cross the cell membrane is inversely proportional to the length of the initial incubation period, a finding which is consistent with the increase in preponderance with time of the less exchangeable storage forms of the drug reported here (Fig. 3 and Table 1). Post-mortem analysis of human liver samples [19] have shown that MTX polyglutamates can persist in this tissue for up to 180 days between the date of the last treatment with the drug and death. This is consistent with the long phase of efflux reported here which results in substantial amounts of drug being retained long after the extracellular drug has been removed (Fig. 4).

In conclusion it is apparent that human breast cancer cells have the ability to retain MTX polyglutamates and this feature of the biochemical pharmacology of MTX may play an important role in determining the response of this tumour to the drug. Our results also suggest that the ability of insulin to potentiate the cytotoxicity of MTX towards human breast cancer cells may be related to the hormone's ability to modify the extent of synthesis of the less exchangeable polyglutamate forms of MTX.

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