Biochemical Pharmacology, Vol. 36, No. 7, pp. 1091–1097, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

METHOTREXATE METABOLISM IN MUTANT CHINESE HAMSTER OVARY CELLS LACKING DIHYDROFOLATE REDUCTASE*†

PILAR JOANNON,‡ V. MICHAEL WHITEHEAD,§ DAVID S. ROSENBLATT, MARY-JANE VUCHICH and DENISE BEAULIEU

The Penny Cole Hematology Research Laboratory, and the Medical Research Council Genetics Group, the McGill University–Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada

(*Received* 19 *May* 1986; *accepted* 25 *August* 1986)

Abstract—To study the influence of the level of dihydrofolate reductase (DHFR) on methotrexate (MTX) metabolism, the formation of methotrexate polyglutamates (MTXPGs) and the retention of the drug were examined in Chinese hamster ovary cells (DUKXB11) lacking DHFR and in control cells (CHO-UTC). Both cells accumulated MTXPGs poorly. After a 24-hr incubation with 1.0 μM [^{3}H]MTX, the level of total MTX in DUKXB11 cells was 40% of that in CHO-UTC cells, reflecting the lack of DHFR-bound MTX and MTXPGs in the mutant cells. MTXPGs accounted for a higher proportion of the intracellular MTX in DUKXB11 than in CHO-UTC cells (25 vs 18%). Following exposure to 3.0 μM MTX for 24 hr, total drug levels were similar in both cell lines, and MTXPGs constituted even more of the intracellular drug in DUKXB11 cells compared to CHO-UTC cells (34 vs 23%). DUKXB11 cells accumulated longer MTXPGs ($MTXG1u_{3,4}$) compared to CHO-UTC cells ($MTXG1u_{2,3}$), following exposure to both 1.0 and 3.0 μM MTX. The longer MTXPGs in the mutant cells may have resulted from the lack of DHFR in them. Binding of MTXPGs to DHFR in CHO-UTC may interfere with their further polyglutamylation. When cells were resuspended in drug-free buffer for 1 hr following a 24-hr incubation with MTX, the retention of drug was less in DUKXB11 cells (46%) than in CHO-UTC cells (78%), due mainly to a greater loss of unmetabolized MTX in the mutant cells (89%) than in control cells (26%). Nevertheless, the amount of non-exchangeable unmetabolized MTX retained in DUKXB11 cells following exposure to 3.0 μM MTX exceeded the MTX-binding capacity. These studies demonstrate that DHFR-deficient cells accumulated more and longer MTXPGs than control cells. In addition, they suggest that some unmetabolized MTX was retained in cells not bound to DHFR.

MTX‖ metabolism to poly-γ-glutamyl derivatives has been demonstrated in a wide variety of cells, including human [1–5] and animal tissues [1, 6–11]. MTXPG formation has been shown to be critical for MTX cytotoxicity [12–18]. MTXPGs, particularly of long-chain length, have a prolonged retention in the cell in the absence of extracellular drug [9, 10, 12–14, 19, 20]. They bind to the target enzyme DHFR with an affinity equal to [4, 7, 10, 12, 21] or greater than [22] that of MTX, and they have a slower dissociation from the enzyme compared to the parent drug [20]. These pharmacologic properties of MTXPGs account for the prolonged inhibition of the synthesis of thymidylate [12–15] and of cell growth [12, 13, 15] after removal of extracellular drug.

Since the formation of MTXPGs plays a determinant role in the cytotoxic action of the drug, the mechanisms by which cells control the synthesis of MTXPGs have been the object of intense research. Several factors that regulate their formation have been identified. Synthesis of MTXPGs is dose and time dependent [3–5, 13, 17], differs from one tissue to another [6, 11], and is increased in cells in logarithmic growth [3, 23, 24].

Previous studies in this laboratory demonstrated that mutant MTX-resistant baby hamster kidney cells, characterized by a high level of DHFR, accumulate high levels of MTX, but very low levels of MTXPGs compared to control cells [25]. These findings suggested that DHFR and FPGS compete for MTX entering the cell and that MTX bound to DHFR is not a substrate for MTXPG synthesis. In addition, it was reported that pretreatment of cultured Ehrlich ascites tumor cells with metoprine [2,4 - diamino - 5 - (3′,4′ - dichlorophenyl) - 6 - methylpyrimidine] to inhibit binding of aminopterin to DHFR, and thereby to increase the free intracellular drug level, results in greater formation of aminopterin polyglutamates [26].

* This work was supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada (V. M. W.), and the Medical Research Council of Canada Genetics Group (D. S. R.). Support was also provided by the Lamplighters Leukemia Association, the Inter Service Clubs Council Telethon of Stars, the Penny Cole Fund, the Fast Foundation, the Debbie Saunders Fund, and friends of Ande.

† This is publication No. 86018 from the McGill University–Montreal Children's Hospital Research Institute.

‡ Research Fellow of the Cancer Research Society and the McGill University–Montreal Children's Hospital Research Institute.

§ To whom correspondence and requests for reprints should be addressed at: Division of Hematology, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Quebec, Canada H3H 1P3.

‖ Abbreviations: MTX, methotrexate (4-deoxy-4-NH_2-10-CH_3PteGlu); DHFR, dihydrofolate reductase, EC 1.5.1.3; MTXPG, methotrexate polyglutamate; $MTXGlu_{2-6}$, MTXPG containing 2 to 6 glutamyl residues; CHO, Chinese hamster ovary; FPGS, folate polyglutamate synthetase; and TBAP, tetrabutylammonium phosphate.

Recently it was shown that levels of DHFR in fresh human tissues, both malignant and normal, are much lower than those found in animals and also in cells in culture, both of human and animal origin [27]. To examine further the influence of DHFR on the metabolism of MTX, we have studied the formation of MTXPGs and the retention of MTX and MTXPGs in mutant Chinese hamster ovary cells lacking DHFR [28]. In addition, this cell line allowed us to observe whether unmetabolized MTX can be retained in cells when not bound to DHFR.

MATERIALS AND METHODS

Chemicals. [3′,5′-7-^{3}H]MTX (specific activity 18 and 20 Ci/mmole) was obtained from Moravek Biochemicals, Inc. (Brea, CA) and was purified by Sephadex G-15 gel chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden) [6]. The specific activity of the radiolabeled drug was adjusted to 6 or 6.6 Ci/mmole by the addition of unlabeled MTX (Lederle Laboratories, Cyanamid Canada Inc., Montreal, Quebec). Modified Ham's F12 medium and Hanks' balanced salt solution were obtained from Flow Laboratories (McLean, VA) and fetal calf serum from Gibco Laboratories (Grand Island, NY). Authentic standards of 4-NH_2-10-CH_3PteGlu$_2$ to Glu$_6$ were provided by Dr. C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile, AL) [29].

Acetonitrile (CH_3CN) was purchased from the Fisher Scientific Co. (Fair Lawn, NJ), tetrabutylammonium phosphate (TBAP) from the Eastman Kodak Co. (Rochester, NY) and Ready-Solv HP liquid scintillation fluid from Beckman Instruments, Inc. (Fullerton, CA). All other chemicals were of reagent or HPLC grade.

Cell cultures. The studies were performed on mutant CHO cells lacking DHFR (DUKXB11), obtained from Dr. Lawrence Chasin [28] and in a control CHO line, a proline auxotroph obtained from Dr. W. F. Flintoff [30]. Both cell lines were grown in monolayer culture, in modified Ham's F12 medium containing glycine, hypoxanthine and thymidine, for which the DHFR-deficient mutant is auxotrophic. The medium was supplemented with 10% fetal calf serum and 4 mM L-glutamine. Cell counting was done in a Coulter Counter model Z_F (Coulter Electronics Inc., Hialeah, FL) after detaching the cells from the flasks with 0.25% trypsin. Cells were plated at an initial density of 1.5×10^6 cells/10 ml on a 100 mm Petri dish and incubated at 37°, under an atmosphere of 5% CO_2–95% air, for about 24 hr before experiments were begun, to allow growth to be underway.

Intracellular accumulation of MTX and MTXPGs. Cells in logarithmic growth were exposed to 1.0 or 3.0 μM [^{3}H]MTX in 5 ml of modified Ham's F12 medium for the times indicated. At the end of the incubation period, the cells were rinsed rapidly three times with 10 ml of ice-cold Hanks' balanced salt solution (pH 7.4).

For measurement of total intracellular MTX, the cells were scraped off the dish surface with a rubber policeman in 2 ml of the same solution. To measure non-exchangeable MTX and MTXPGs, rinsed cells were incubated for an additional 1 hr in 5 ml of Hanks' solution (without MTX) [14] and then removed from the dishes as described above. After harvesting, cells were frozen and stored overnight at −20° or processed as follows. After thawing, cells were further disrupted by sonication (twice for 15 sec) using a sonic dismembrator (Fisher Co.). Aliquots were taken to measure total radioactivity and protein concentration of cell extracts. Then the protein was precipitated with 10% trichloroacetic acid, followed by centrifugation at 3500 *g* for 10 min.

The sample was concentrated by passage through a Sep-Pak C18 cartridge (Waters Associates Inc., Milford, MA) [13, 31]. The Sep-Pak was pre-rinsed with 2 ml of 100% methanol followed by 5 ml water, 5 ml of solution A (5 mM TBAP and 10 mM KH_2PO_4, pH 5.5) and 5 ml water. The supernatant fraction was absorbed into the Sep-Pak, washed with 5 ml water, and MTX and its derivatives were eluted with 2 ml of 100% methanol. The eluate was dried under nitrogen and dissolved in 200 μl of solution A. Recoveries following Sep-Pak concentration and drying were 82.7 ± 6.4% of the radioactivity in the cell extract. There was no preferential loss of MTXPG standards during passage through the Sep-Pak.

Separation of MTX and MTXPGs was performed by HPLC using a Radial-Pak C_8 column (Waters Associates) as described by Jolivet *et al.* [13]. The sample was eluted at 2 ml/min along a linear gradient of 20–29% CH_3CN in solution A over 15 min, followed by increasing the concentration of CH_3CN from 29 to 36% over the subsequent 25 min using an Automated Gradient Controller (Waters Associates), and an M-45 and a 6000A Solvent Delivery System (Waters Associates). Fractions of 1 ml were collected and radioactivity was counted on a 1215 Rack-Beta Liquid Scintillation Counter (LKB Wallac).

MTX and MTXPGs were identified by co-chromatography with authentic standards (4-NH_2-10-CH_3PteGlu$_1$ to -Glu$_6$), which were injected in a 2-μl volume with the sample. Their positions were determined by monitoring u.v. absorbance at 313 nm (model 441 detector, Waters Associates). The mean recovery of labeled MTX and MTXPGs was 89.9 ± 17.1% of the radioactivity injected on the column. The chromatograms showed some ^{3}H-labeled material that did not co-chromatograph with the authentic standards, and whose amount varied from one experiment to another. This undetermined material accounted for 22.5 ± 9.7% of the total ^{3}H of the injected sample. The total amount of drug in the cell was calculated by subtracting the percent of contaminants of each sample from the total ^{3}H of the cell extract. The intracellular distribution of MTX was calculated considering the percentage of each ^{3}H-labeled fraction determined by HPLC, and the total amount of drug in the cell. The results were expressed as nmoles of MTX and MTX derivatives per g of protein ± one standard deviation. The protein concentration of the cell extracts was determined by the method of Lowry *et al.* [32].

MTX-binding capacity. The level of DHFR in the cells was measured by [^{3}H]MTX binding [33]. A standard of mammalian DHFR was obtained from

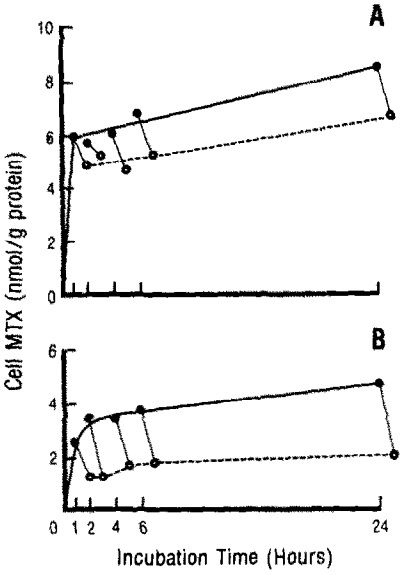

Fig. 1. Total and non-exchangeable MTX in CHO-UTC and DUKXB11 cells. CHO-UTC (A) and DUKXB11 (B) cells were incubated in culture medium containing 1.0 μM [^{3}H]MTX for 1, 2, 4, 6 and 24 hr. Then the cells were rinsed three times with ice-cold Hanks' solution, and the total intracellular MTX was determined (●——●). Replicate plates were incubated for the same time intervals, rinsed, and then incubated in MTX-free buffer for 1 hr [non-exchangeable MTX (○---○)]. Each point represents the mean of duplicate measurements.

MTX-resistant mutant baby hamster kidney cells [25]. Aliquots of cytoplasmic extracts were incubated with from 3.0 to 6.0 nM [^{3}H]MTX for 10 min at 4° in the presence of excess NADPH, followed by adsorption of the unbound [^{3}H]MTX with dextran T-10 coated charcoal.

RESULTS

Growth of CHO cells. DUKXB11 cells grew at a slower rate than CHO-UTC cells; the doubling times were 23 ± 3 and 17 ± 2 hr respectively (N = 6) ($P < 0.0005$). Increasing the concentration of thymidine and hypoxanthine in the growth medium did not increase DUKXB11 cell growth. As expected, cell growth was not affected by MTX, since both control and mutant cells were grown in medium containing end products of folate metabolism.

DHFR levels in CHO-UTC and DUKXB11 cells. The level of DHFR, measured by MTX binding capacity, was more than 2 logs lower in the mutant DHFR-deficient cells than in the wild-type. The MTX-binding capacity of CHO-UTC cells was 6.20 ± 1.06 nmoles/g (N = 12), whereas that of DUKXB11 cells was 0.05 ± 0.03 nmoles/g (N = 3). The extremely low MTX binding capacity of the DUKXB11 cytoplasmic extract reflects the lack of DHFR in this mutant cell line.

Intracellular accumulation of total and of non-exchangeable MTX. Drug levels accumulated in the cells after exposure to 1.0 μM [^{3}H]MTX for various periods of time over a 24-hr interval were determined. As shown in Fig. 1, there was an initial rapid uptake of MTX. A steady-state level was achieved after 1–2 hr of incubation, with a further modest increase by 24 hr in both cell lines. Levels of total intracellular MTX were lower in DUKXB11 than in CHO-UTC cells. When the cells were incubated in drug-free buffer for 1 hr following the various times of incubation with MTX, DUKXB11 cells retained a lower proportion of the total intracellular MTX compared to CHO-UTC cells. As shown in Table 1, the total MTX level in the DUKXB11 cells after 24 hr of incubation with 1.0 μM MTX was 40% of that in CHO-UTC cells ($P < 0.0005$). The level of non-exchangeable MTX in the mutant cells after a 1-hr efflux following a 24-hr incubation with the drug was only 23% of that in control cells ($P < 0.0005$).

MTXPG formation. Following a 24-hr exposure of both cell lines to 1.0 μM [^{3}H]MTX, MTXPGs comprised 18 and 25% of the total intracellular MTX in CHO-UTC and DUKXB11 cells respectively (Table 1). Despite the fact that DUKXB11 cells accumulated less total intracellular MTX, a higher proportion of drug was present as MTXPGs. However, in absolute terms the amount of MTXPGs accumulated in the DUKXB11 cells was lower than in CHO-UTC cells ($P < 0.01$), since the total intracellular MTX was lower in the mutant cells (Table 1).

To increase the intracellular drug accumulated in DUKXB11 cells, the level of extracellular MTX was increased to 3.0 μM. As shown in Table 2, the total intracellular MTX accumulated in DUKXB11 cells increased and became comparable to that accumulated in CHO-UTC cells exposed to either 1.0 or 3.0 μM MTX. The increase in extracellular concentration of MTX appears to have resulted in a greater accumulation of MTXPGs in the DUKXB11 cells than in CHO-UTC cells. MTXPGs represented a greater proportion of total intracellular MTX in DUKXB11 cells than in CHO-UTC cells ($P < 0.0005$).

Intracellular distribution of MTX and MTXPGs. The distribution of total MTX and MTXPGs following exposure of both CHO cell lines to 1.0 and 3.0 μM radiolabeled drug for 24 hr is illustrated in Fig. 2. With both concentrations, $MTXGlu_2$ and $MTXGlu_3$ were the predominant forms of MTXPGs in CHO-UTC cells. In contrast, DUKXB11 cells formed mainly $MTXGlu_3$ and $MTXGlu_4$. As shown in Table 1, following a 24-hr exposure to 1.0 μM MTX, DUKXB11 cells accumulated a very low level of $MTXGlu_2$ compared to CHO-UTC cells ($P < 0.0025$). On the other hand, the level of $MTXGlu_4$ in DUKXB11 cells was 10-fold higher than in CHO-UTC cells ($P < 0.0005$). In addition, $MTXGlu_5$ was detected in the mutant cells. As shown in Table 2, after exposure to 3.0 μM MTX, the chain length of the MTXPGs increased in both mutant and control cells. Longer MTXPGs were again observed in the mutant cells, which accumulated small amounts of $MTXGlu_6$. In addition, with the higher extracellular concentration of MTX, the differences observed in $MTXGlu_2$ and $MTXGlu_4$ levels between these two cell lines remained significant ($P < 0.005$ and $P < 0.0005$ respectively).

Composition of non-exchangeable MTX and derivatives. Exposure of the cells to 1.0 μM MTX for 24 hr, followed by incubation in drug-free buffer

Table 1. Total and non-exchangeable MTX and MTXPG levels in CHO-UTC and DUKXB11 after incubation of cells in 1.0 μM [^{3}H]MTX for 24 hr

Cell line	Time of efflux	Intracellular MTX and MTX derivatives (nmoles/g)						
		Total MTX	MTX	MTX Glu_2	MTX Glu_3	MTX Glu_4	MTX Glu_5	Total MTXPGs
CHO-UTC		6.59 ± 0.39	5.42 ± 0.26	0.55 ± 0.21	0.59 ± 0.22	0.03 ± 0.03		1.17 ± 0.28
			(82 ± 4)*	(8 ± 3)	(9 ± 3)	(0.5 ± 0.5)		(18 ± 4)
CHO-UTC	1hr	5.14 ± 0.59	3.99 ± 0.33	0.52 ± 0.03	0.61 ± 0.26	0.02 ± 0.02		1.15 ± 0.30
			(78 ± 3)	(10 ± 1)	(12 ± 4)	(0.5 ± 0.3)		(22 ± 3)
DUKXB11		2.61 ± 0.21	1.97 ± 0.26	0.02 ± 0.03	0.31 ± 0.02	0.29 ± 0.04	0.02 ± 0.01	0.64 ± 0.08
			(75 ± 4)	(1 ± 1)	(12 ± 1)	(11 ± 2)	(0.8 ± 0.3)	(25 ± 4)
DUKXB11	1 hr	1.20 ± 0.28	0.21 ± 0.11		0.42 ± 0.06	0.50 ± 0.11	0.07 ± 0.03	0.99 ± 0.18
			(17 ± 6)		(36 ± 5)	(41 ± 3)	(6 ± 1)	(83 ± 6)

Following a 24-hr incubation in culture medium containing 1.0 μM [^{3}H]MTX, cells were rinsed three times with ice-cold Hanks' solution, and the cell extracts were analyzed for radioactivity (total intracellular MTX). Cells in replicate plates were rinsed, resuspended in MTX-free buffer for 1 hr and extracted (non-exchangeable MTX). The intracellular distribution of MTX and MTXPGs was determined by HPLC as described in Materials and Methods. Each value represents the mean ± one standard deviation (N = 4).

* Percent of total MTX.

Table 2. Total and non-exchangeable MTX and MTXPG levels in CHO-UTC and DUKXB11 after incubation of cells in 3.0 μM [^{3}H]MTX for 24 hr

Cell line	Time of efflux	Intracellular MTX and MTX derivatives (nmoles/g)							
		Total MTX	MTX	MTX Glu_2	MTX Glu_3	MTX Glu_4	MTX Glu_5	MTX Glu_6	Total MTXPGs
CHO-UTC		6.82 ± 1.15	5.27 ± 0.93	0.62 ± 0.26	0.74 ± 0.07	0.12 ± 0.02	0.07 ± 0.04		1.55 ± 0.29
			(77 ± 2)*	(9 ± 3)	(11 ± 1)	(2 ± 0.5)	(1 ± 0.6)		(23 ± 2)
CHO-UTC	1 hr	4.52 ± 0.66	3.10 ± 0.40	0.52 ± 0.19	0.72 ± 0.17	0.09 ± 0.03	0.09 ± 0.05		1.42 ± 0.30
			(69 ± 3)	(11 ± 3)	(16 ± 2)	(2 ± 0.7)	(2 ± 1.6)		(31 ± 3)
DUKXB11		5.90 ± 0.77	3.90 ± 0.54	0.24 ± 0.06	0.92 ± 0.15	0.68 ± 0.08	0.13 ± 0.09	0.03 ± 0.07	2.00 ± 0.30
			(66 ± 3)	(4 ± 1)	(16 ± 2)	(12 ± 2)	(2 ± 1)	(0.04 ± 1)	(34 ± 3)
DUKXB11	1 hr	2.32 ± 0.63	0.45 ± 0.18	0.14 ± 0.05	0.79 ± 0.29	0.72 ± 0.21	0.17 ± 0.13	0.05 ± 0.13	1.87 ± 0.46
			(19 ± 3)	(6 ± 1)	(34 ± 7)	(31 ± 5)	(8 ± 7)	(3 ± 6)	(81 ± 3)

The cells were incubated for 24 hr with 3.0 μM [^{3}H]MTX. The total and non-exchangeable MTX and MTXPGs in the cell were determined as described in Table 1. Results represent means ± one standard deivation (N = 6).

* Percent of total MTX.

for an additional 1 hr, resulted in a lower retention of total MTX in the DUKXB11 cells than in the CHO-UTC cells (46 vs 78%) (Table 1). This difference was due to a much lower retention of unmetabolized MTX in DUKXB11 cells compared to CHO-UTC cells (11 vs 74%). MTXPG pools remained essentially the same after 1 hr efflux in CHO-UTC, but increased in DUKXB11 cells. When cells were incubated with 3.0 μM MTX for 24 hr, followed by incubation in drug-free buffer for 1 hr, the fall in unmetabolized MTX was again greater in DUKXB11 than in CHO-UTC cells (12 vs 59%). There was no measurable change in quantity or distribution of MTXPGs in either cell line (Table 2).

The level of exchangeable MTX after a 24-hr incubation with 1.0 μM MTX was similar in both cell lines (1.45 nmoles/g in CHO-UTC and 1.41 nmoles/g in DUKXB11 cells) (Table 1). With 3.0 μM MTX, the level of exchangeable MTX in DUKXB11 cells was higher than in CHO-UTC cells (3.58 vs 2.30 nmoles/g) (Table 2). Thus, the amount of MTX not bound to DHFR increased more in DUKXB11 cells following exposure to 3.0 μM MTX than in CHO-UTC.

Retention of MTX in DUKXB11 cells. The levels of unmetabolized MTX retained after 1-hr efflux in DUKXB11 cells incubated with 1.0 and 3.0 μM MTX were 0.21 ± 0.11 and 0.45 ± 0.18 nmoles/g respectively, both greater than the MTX-binding capacity of the cell supernatant fraction (0.05 ± 0.03 nmole/g protein) measured in that cell line by [^{3}H]MTX binding.

To determine whether these levels of non-exchangeable unmetabolized MTX in DUKXB11 cells reflected a slow rate of loss of MTX, the time course of efflux of MTX was studied in DUKXB11

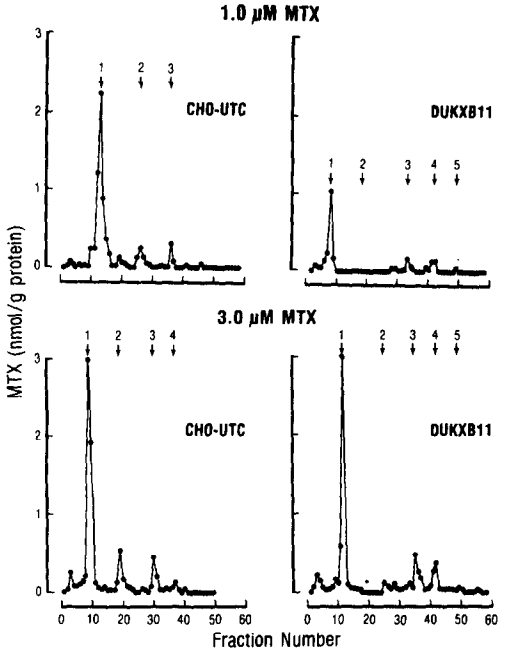

Fig. 2. Accumulation of total MTX and MTXPGs in CHO-UTC and DUKXB11 cells. Shown are HPLC chromatograms of CHO-UTC and DUKXB11 cell extracts after incubation of the cells with 1.0 and 3.0 μM [^{3}H]MTX for 24 hr. Arrows numbered 1–5 represent the position of elution of MTXGlu$_{1-5}$ standards respectively.

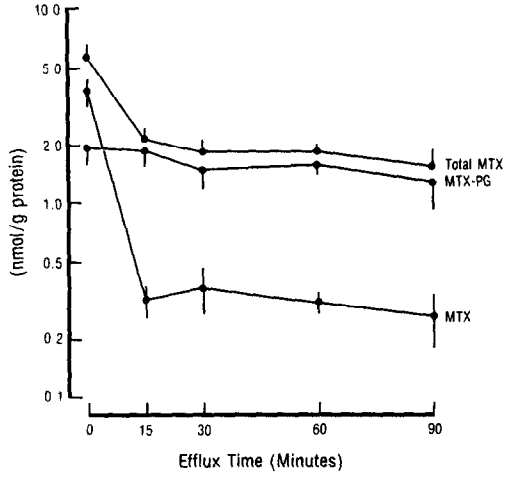

Fig. 3. The non-exchangeable MTX pools as a function of time of efflux. DUKXB11 cells were incubated with 3.0 μM [^{3}H]MTX for 24 hr, rinsed three times with ice-cold Hanks' solution, and incubated for 15, 30, 60 and 90 min in MTX-free buffer. Results represent the mean ± one standard deviation (N = 4). Note that the level of MTX remaining after efflux exceeds the MTX-binding capacity of the cell supernatant fraction (0.05 ± 0.03 nmole/g protein).

cells following 24-hr incubation with 3.0 μM MTX. As shown in Fig. 3, loss of intracellular MTX was greatest during the first 15 min of efflux after which a constant level of MTX was maintained. Most of the drug loss was due to efflux of unmetabolized MTX, whereas the MTXPG pool did not change (Fig. 3). The level of non-exchangeable unmetabolized MTX in DUKXB11 cells was higher than the level of DHFR in these cells throughout the efflux period studied.

DISCUSSION

The present study describes the metabolism of MTX in mutant CHO cells lacking DHFR compared to control cells. The extremely low level of DHFR detected in DUKXB11 cells was similar to the value reported previously from this laboratory [34]. Since a very concentrated cell extract was needed to detect [^{3}H]MTX binding, results may, in part, reflect non-specific binding. In addition, since the counts obtained were only twice background, the true level of DHFR in DUKXB11 may be even lower than reported here. The DHFR level found in the CHO-UTC cells was slightly higher than that previously reported from this laboratory [34], but in agreement with that reported by Urlaub and Chasin [28].

Both control and mutant CHO cells accumulated MTX rapidly with little additional increase after the first 1–2 hr of incubation (Fig. 1). This suggests that the level of DHFR did not increase greatly during incubation with MTX. Non-exchangeable MTX also did not increase much with prolonged incubation. These results reflect the low capacity of these CHO cells to accumulate MTXPGs. By contrast, H-35 hepatoma cells [8] showed an extended linear uptake of MTX with extensive conversion of MTX to MTXPGs. Similarly, human fibroblasts [14] and Ehrlich ascites tumor cells [10] demonstrated an increase of non-exchangeable MTX with time of incubation that paralleled the rise in MTXPGs.

Compared to other cells reported in the literature, including H-35 rat hepatoma cells [8], rat hepatocytes [9], human fibroblasts [3], human breast cancer cells [4, 13], and leukemic cells [5, 35, 36], CHO cells synthesized MTXPGs poorly, despite careful precautions to prevent their *in vitro* hydrolysis. The explanation of this phenomenon is not yet clear. Several factors are known to contribute to low MTXPG formation. These include decreased uptake of MTX, increased intracellular folates, reduced activity of FPGS, or increased hydrolysis of MTXPGs by γ-glutamyl hydrolases. Previous studies have shown that the accumulation of MTX differs among different tissues [6, 11]. Moreover, it has been suggested that tumor cells synthesize more MTXPGs than normal tissues [11]. These differences in the accumulation of MTXPGs appear to be an important element in MTX selectivity and cytotoxicity [16].

DUKXB11 cells accumulated lower amounts of total MTX than control cells. The slower growth rate of DUKXB11 may partially explain this observation. More likely, it represents the lack of MTX and MTXPGs bound to DHFR in the mutant cells. Previous studies showed that exposure of cells containing a high level of DHFR, such as L1210 mouse leukemia cells [7], and MTX-resistant baby hamster kidney cells [25] to MTX results in the accumulation of very high levels of intracellular drug, not due to the synthesis of MTXPGs but to drug bound to DHFR.

DUKXB11 cells accumulated proportionately more MTXPGs than control cells. This greater synthesis of MTXPGs in cells lacking DHFR may have been the result of a higher level of free intracellular MTX in DUKXB11 cells. Previous studies in this laboratory have suggested that MTX bound to DHFR is not a substrate for MTXPG synthesis [7, 25]. Other studies have suggested that exchangeable MTX is required for polyglutamate synthesis [9]. In this regard, our results showed that exchangeable MTX, representing MTX not bound to DHFR, increased in the DUKXB11 cells following exposure to 3.0 μM compared with 1.0 μM MTX and was associated with a greater accumulation of MTXPGs.

Several studies have demonstrated that slowly proliferating cells accumulate lower amounts of MTXPGs [3, 23, 24]. In this study, other factors already mentioned played a more important role, since DUKXB11 cells, despite a slower growth rate, formed greater amounts of MTXPGs. Previous studies from this laboratory failed to demonstrate that DUKXB11 cells form greater amounts of MTXPG than control cells [34]. These studies used a 2-hr incubation in 1.0 μM MTX, conditions now known to be unlikely to show a difference.

It is of interest that DUKXB11 cells formed MTXPGs of longer chain-length than did control CHO-UTC cells after incubation with either 1.0 or 3.0 μM MTX and despite their slower growth rate. Previous studies have reported formation of longer MTXPGs in relation to lower extracellular drug concentrations [37], and in cells with rapid growth [24].

We believe the accumulation of longer MTXPGs in the DUKXB11 cells is due mainly to their lack of DHFR. Previous studies have shown that MTXPGs, once formed, replace unmetabolized MTX on DHFR [10, 12, 20]. In mutant cells, both MTX and MTXPGs constituted a substrate pool for polyglutamate synthesis. By contrast, some of the MTXPGs formed in CHO-UTC cells would bind to DHFR and in that state would not be further polyglutamylated. The greater retention of $MTXGlu_2$ in CHO-UTC cells after efflux suggests that it is bound to DHFR.

These results have a bearing on MTX metabolism in fresh human tissues, which have been found to have a lower level of DHFR than animal tissues and cells in culture [27]. Low levels of DHFR may contribute to the extensive synthesis of MTXPGs in lymphoblasts from children with acute lymphoblastic leukemia. In these cells, the predominant species was $MTXGlu_5$. (V. M. Whitehead, D. S. Rosenblatt, M. J. Vuchich and D. Beaulieu, unpublished observation).

The present study shows that DHFR plays an important role in the retention of MTX in cells. When cells were resuspended in drug-free buffer following incubation with MTX, DUKXB11 retained a lower proportion of drug, due to a greater loss of unmetabolized MTX. Unmetabolized MTX rapidly leaves cells after removal of the extracellular drug (Fig. 3). Of interest was the finding that DUKXB11 cells retained unmetabolized MTX in excess of their measured MTX binding capacity. This was particularly evident following exposure to 3.0 μM MTX (Table 2). Efflux studies showed that loss of unmetabolized MTX was rapid and confirmed that the level of unmetabolized MTX retained in the cell exceeded the MTX-binding capacity (Fig. 3). These findings show that some unmetabolized MTX was indeed retained in DUKXB11, rather than that apparent retention was due to slow drug efflux.

It has been reported that MTX increases DHFR levels in cultured cells as well as in cells of patients treated with the drug [20, 38–40]. Although it is possible that an increase of DHFR-binding capacity occurred during 24-hr incubation of cells with MTX, it is unlikely because the mutant cells have a lesion at the DHFR locus [27]. In addition, total MTX levels did not increase progressively over 24 hr in CHO-UTC cells exposed to MTX (Fig. 1). It seems likely therefore that DUKXB11 cells retain some unmetabolized MTX bound to the cell membrane or within cell particles. Further studies are necessary to clarify this observation.

Acknowledgements—We thank Dr. J. Jolivet for assistance with HPLC methodology, N. V. Matiaszuk for assistance with tissue culture, and L. Lam Han for assistance in the preparation of the manuscript.

REFERENCES

1. C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. biophys. Res. Commun.* **52**, 27 (1973).
2. S. A. Jacobs, C. J. Derr and D. G. Johns, *Biochem. Pharmac.* **26**, 2310 (1977).
3. D. S. Rosenblatt, V. M. Whitehead, M. M. Dupont, M-J. Vuchich and N. Vera, *Molec. Pharmac.* **14**, 210 (1978).
4. R. L. Schilsky, B. D. Bailey and B. A. Chabner, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2919 (1980).
5. A. Witte, V. M. Whitehead, D. S. Rosenblatt and M-J. Vuchich, *Devl. Pharmac. Ther.* **1**, 40 (1980).
6. V. M. Whitehead, M. M. Perrault and S. Stelcner, *Cancer Res.* **35**, 2985 (1975).
7. V. M. Whitehead, *Cancer Res.* **37**, 408 (1977).
8. J. Galivan, *Cancer Res.* **39**, 735 (1979).
9. D. A. Gewirtz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.* **39**, 2914 (1979).
10. D. W. Fry, J. C. Yalowich and I. D. Goldman, *J. biol. Chem.* **257**, 1890 (1982).
11. R. G. Poser, F. M. Sirotnak and P. L. Chello, *Cancer Res.* **41**, 4441 (1981).
12. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
13. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
14. D. S. Rosenblatt, V. M. Whitehead, N. Vera, A. Pottier, M. Dupont and M-J. Vuchich, *Molec. Pharmac.* **14**, 1143 (1978).
15. D. S. Rosenblatt, V. M. Whitehead, N. Vera-Matiaszuk, A. Pottier, M-J. Vuchich and D. Beaulieu, *Molec. Pharmac.* **21**, 718 (1982).
16. I. Fabre, G. Fabre and I. D. Goldman, *Cancer Res.* **44**, 3190 (1984).
17. S. Koizumi, G. A. Curt, R. L. Fine, J. D. Griffin and B. A. Chabner, *J. clin. Invest.* **75**, 1008 (1985).
18. K. H. Cowan and J. Jolivet, *J. biol. Chem.* **259**, 10793 (1984).
19. M. Balinska, J. Galivan and J. K. Coward, *Cancer Res.* **41**, 2751 (1981).
20. J. Jolivet and B. A. Chabner, *J. clin. Invest.* **72**, 773 (1983).
21. S. A. Jacobs, R. H. Adamson, B. A. Chabner, C. J. Derr and D. E. Johns, *Biochem. biophys. Res. Commun.* **63**, 692 (1975).

22. B. A. Chabner, C. J. Allegra, G. A. Curt, N. J. Clendeninn, J. Baram, S. Koizumi, J. C. Drake and J. Jolivet, *J. clin. Invest.* **76**, 907 (1985).
23. Z. Nimec and J. Galivan, *Archs. Biochem. Biophys.* **226**, 671 (1983).
24. D. G. Kennedy, H. W. Van den Berg, R. Clarke and R. F. Murphy, *Biochem. Pharmac.* **34**, 3087 (1985).
25. V. M. Whitehead and D. S. Rosenblatt, in *Chemistry and Biology of Pteridines* (Eds. R. L. Kisliuk and G. M. Brown), p. 689. Elsevier/North Holland, New York (1979).
26. L. H. Matherly, M. K. Voss, L. A. Anderson, D. W. Fry and I. D. Goldman, *Cancer Res.* **45**, 1073 (1985).
27. B. A. Kamen, P. A. Nylen, V. M. Whitehead, H. T. Abelson, B. J. Dolnick and D. W. Peterson. *Cancer Drug Delivery* **2**, 133 (1985).
28. G. Urlaub and L. A. Chasin, *Proc. natn. Acad. Sci. U.S.A.* **77**, 4216 (1980).
29. M. G. Nair and C. M. Baugh, *Biochemistry* **12**, 3923 (1973).
30. W. F. Flintoff, S. V. Davidson and L. Siminovitch, *Somat. Cell Genet.* **2**, 245 (1976).
31. J. Jolivet and R. L. Schilsky, *Biochem. Pharmac.* **30**, 1387 (1981).
32. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
33. B. A. Kamen, P. L. Takach, R. Vatev and J. D. Caston, *Analyt. Biochem.* **70**, 54 (1976).
34. D. S. Rosenblatt and V. M. Whitehead, in *Proceedings of the Second Workshop on Folyl and Anti-Folyl Polyglutamates.* (Ed. I. D. Goldman), p. 224. Praeger Scientific, New York (1985).
35. V. M. Whitehead and D. S. Rosenblatt, *Adv. exp. Med. Biol.* **163**, 287 (1983).
36. V. M. Whitehead and D. S. Rosenblatt, in *Chemistry and Biology of Pteridines and Folic Acid Derivatives* (Ed. J. A. Blair), p. 927. Walter de Gruyter, Hawthorne, NY (1983).
37. M. Balinska, Z. Nimec and J. Galivan, *Archs. Biochem. Biophys.* **216**, 466 (1982).
38. J. R. Bertino, D. M. Donohue, B. Simmons, B. W. Gabrio, R. Silber and F. M. Huennekens, *J. clin. Invest.* **42**, 466 (1963).
39. B. A. Domin, S. P. Grill, K. F. Bastow and Y-C. Cheng, *Molec. Pharmac.* **21**, 478 (1982).
40. B. L. Hillcoat, V. Swett and J. R. Bertino, *Proc. natn. Acad. Sci. U.S.A.* **58**, 1632 (1967).