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Effect of Methotrexate on Dihydrofolate Reductase Activity in Methotrexate-Resistant Human KB Cells

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SUMMARY

A cloned subline of human cell variants derived from KB cells (KB/6b), 6500-fold resistant to methotrexate (MTX), exhibited a stable 40-fold elevation of the enzyme dihydrofolate reductase (EC 1.5.1.3., DHFR) in the absence of the drug. No differences were detected between the enzyme isolated from resistant and parental KB cells. After the culture medium was supplemented with MTX, the resistant cells were shown to have an additional 5-fold increase in DHFR levels. This increment was dependent upon the concentration of exogenous MTX and was freely reversible. This behavior differs from that of other DHFR-overproducing mammalian cell lines previously reported. The half-life of DHFR in these cells in the presence or absence of MTX was the same, thus eliminating stabilization of the enzyme by MTX as being responsible for the modulation or "induction" phenomenon. Furthermore, the effect was blocked by cycloheximide and was not affected by actinomycin D. These results suggest that modulation of DHFR specific activity by MTX occurs at either the translational level or by as yet undefined post-transcriptional mechanism(s).

INTRODUCTION

Increased levels of the enzyme DHFR[1] (EC 1.5.1.3.) in cells resistant to the folate analogue MTX has recently been shown to be a direct result of increased DHFR gene copy number (1). Such resistant variants occur in two distinct types which can be differentiated according to the stability of their phenotypes with respect to the level of DHFR in the absence of selection (1–7). The behavior of unstable variants is consistent with the DHFR genes' being maintained on multiple autonomous genetic elements which are independent of the cellular chromosome complement. Reversion of the resistant phenotype in the absence of selection is accompanied by an irreversible loss of the "double minute chromosomes" bearing the DHFR genes (8, 9). In contrast, the genes in stable amplified cell variants are localized on a unique chromosome and are maintained in the absence of selection (3, 10).

In this communication we describe a cloned human cell variant which exhibits unique behavior since its resistant phenotype has both a stable and an unstable component, the former being independent of selection whereas the latter is dependent on exogenous MTX. This modulation of phenotype in human cells is reminiscent of a previously reported "induction" phenomenon which has been proposed to be due to intracellular stabilization of the enzyme by MTX (11–13). In contrast, a recent report with murine cells has shown no difference in the cellular turnover of DHFR with MTX present (9). Therefore, this study examines the questions of whether there are qualitative differences with respect to kinetic properties between the DHFR isolated from the resistant cell variant and those from the parental cells or whether the increase in specific activity of DHFR following addition of MTX is a consequence of enzyme stabilization or another mechanism requiring concomitant nuclear transcription and/or protein synthesis, and whether the observed increment in DHFR levels is related to the MTX concentration and is freely reversible.

EXPERIMENTAL PROCEDURES

Materials. L-[^{35}S]Methionine (1050 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.), and [3′-5′-7′-^{3}H]MTX was purchased from Amersham Corporation (Arlington Heights, Ill.). Protein A-Staph was purchased from Meloy Laboratories (Springfield, Va.). L1210 DHFR antibody serum was generously provided by Dr. S. Smith of Wellcome Research Laboratories (Research Triangle Park, N. C.) and purified by DEAE-Affi-gel Blue (Bio-Rad Laboratories, Richmond, Calif.) chromatography (procedure described

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[1] The abbreviations used are: DHFR, dihydrofolate reductase; MTX, methotrexate; H_2PteGlu, 5,6,7,8-dihydrofolic acid; SDS, sodium dodecyl sulfate.

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in Bio-Rad Laboratories Product Information Bulletin 1062, April 1978). MTX-Sepharose was graciously provided by Dr. D. Bacchanari of Wellcome Research Laboratories. MTX was a generous gift of Dr. R. L. Capizzi of the University of North Carolina at Chapel Hill.

Cells. The cell line (KB/6b) for this study was a cloned subline of methotrexate-resistant human KB cells growing in monolayer in RPMI 1640 medium containing 5% fetal calf serum, kanamycin (100 μg/ml), 25 μM MTX, and 30 μM thymidine. This cell line is 6500-fold resistant to MTX and has a 40-fold elevation of DHFR (14). Log-phase cells were harvested by scraping them from the flask and washing them with cold phosphate-buffered saline (0.14 M NaCl, 4.0 mM KCl, 0.5 mM $NaHPO_4$, 0.15 mM $KHPO_4$). Extraction of DHFR was as previously described (15) except that the labeled cells used in the DHFR turnover study were not sonicated and the extraction buffer contained 1% Triton X-100.

[^{35}S]Methionine Labeling of cells. KB/6b cells, growing in the presence and absence of MTX, were labeled in methionine-free medium with 2 μCi of [^{35}S]methionine (1050 Ci/mmole for 24 hr at which time they were diluted 1:4 and refed with normal growth medium. After a further 24 hr, the zero-time samples were harvested. MTX was present throughout the experiment for the drug-supplemented cells and absent throughout the experiment for the depleted cells.

Removal of DHFR-bound MTX. Extracts from all cells, in either the presence or absence of MTX, were incubated for 10 min at room temperature with an equal volume of 1 M Tris-HCl (pH 8.5) and 100 μM H_2PteGlu, applied to Sephadex G-25 columns (1.0 × 1.7 cm) at 5°, and eluted with 0.05 M Tris-HCl buffer (pH 8.5) containing 10% glycerol and 2 mM dithiotreitol. Control experiments using cells exposed to [^{3}H]MTX for 4 days showed that the enzyme fraction obtained following this protocol contained about 10% or less of the total radioactivity, presumably MTX polyglutamate. Therefore, this method may have underestimated the enzymatic activity by not more than 10%. In contrast, when the enzyme was estimated by differential pH assays (pH 8.5 and 7.5) as reported previously (11), the observed activity was only 30% of that obtained with the protocol described here. On the basis of this comparison, we used the column technique for evaluating DHFR activities throughout the entire study and these values are reported as actual DHFR. The DHFR activity measured at pH 7.5 in the crude extract is designated as apparent DHFR.

Immunoprecipitation of DHFR. A 15-μl quantity of 10% saturated deoxycholate, 15 μl of 10% Triton X-100, and 25 μl of crude cellular extract were preincubated at 25° for 10 min. A portion of the immunoglobulin G fraction of L1210 DHFR antibody (125 μl) was added and incubation was continued for 30 min. Preimmune rabbit serum was used in controls. Protein A-Staph (200 μl) was added and the precipitate was centrifuged in a Beckman Microfuge, washed twice with 50 mM Tris-HCl buffer, solubilized in 0.2 ml of 10% SDS and 0.8 ml of the above buffer, and counted in a toluene-based scintillant. Fractionation of the solubilized precipitate on SDS gels followed by autoradiography showed only one band whose mobility corresponded to the purified DHFR protein. Enzyme purification, enzyme assays, and K_i determination for MTX were carried out as previously described (15).

RESULTS

A comparative analysis of the properties of both parental KB cells, the MTX-resistant subline 6b, and the purified DHFR isolated from these two lines is shown in Table 1. The KB/6b subline had a 40-fold elevation of DHFR (in the absence of MTX), and this increase was accompanied by a 6500-fold resistance to MTX (ID_{50} of 65 μM). The enzyme from the resistant cells was identical with that from the parental cells in the kinetic parameters of K_m for H_2PteGlu and NADPH, turnover number per MTX binding site, and K_i for MTX, suggesting no obvious differences between the two enzymes.

Following the removal of MTX from KB/6b cells, the actual DHFR specific activity decreased as shown in Fig. 1. The actual specific activity of DHFR in the presence of MTX was 1.4 units/mg of protein and this decreased to a plateau level by Day 10 (*solid line*). The *dotted line*, representing the ratio of apparent DHFR activity, as measured by pH 7.5 assay of the crude extract, to the actual activity after removal of MTX from the same extract, concomitantly increased to 100%, thus indicating that no MTX was present. The plateau level of DHFR, which is 40-fold higher than that of KB parental cells, remained stable in cells growing in the absence of MTX for more than 1 year. This is different from the behavior of DHFR activity on removal of MTX in other reported "stable" murine resistant cell lines (3, 4, 16).

Earlier reports (11–13, 17) suggested that MTX may increase enzyme activity by stabilizing DHFR in cells.

TABLE 1

Comparative properties of parental and MTX-resistant KB/6b cells and of their purified DHFR

Enzymes were purified on MTX-sepharose as previously described (15). The K_i for MTX was determined by the method of Cha (18) and Cha *et al.* (19) as previously described (15).

Cell line	Degree	Kinetic properties of DHFR			
		K_m H_2PteGlu	K_m NADPH	Turnover no. per MTX binding site	K_i MTX
	units/mg	*-fold*	*μM*	*μM*		*pM*
KB	0.007	1	0.7	5.9	1000	5
KB/6b	0.284	6500	0.7	5.9	900	7

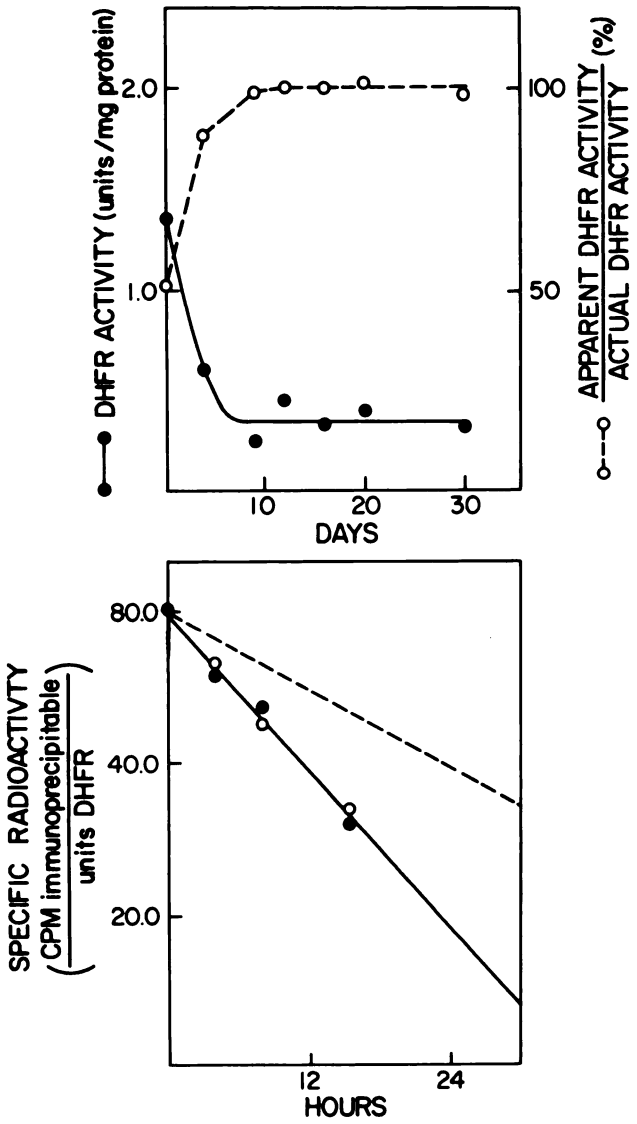

FIG. 1. *Stability of DHFR activity after removal of methotrexate from KB/6b cells and half-life of DHFR in cells in the presence and absence of MTX*

The *upper portion* shows the actual DHFR specific activity after removal of MTX in KB/6b cells. Cells were harvested and DHFR was extracted and assayed as described under Methods. ● Represents units of actual DHFR per milligram of protein, and ○ represents the ratio of apparent DHFR (measurement at pH 7.5 in the crude extract) to the actual DHFR (measurement of the same extract after removal of MTX as described under Methods) expressed as percentage.

The *lower portion* shows the decay in specific radioactivity of DHFR in the presence (●) and absence (○) of MTX with time. Cells were labeled with [^{35}S]methionine as described under Methods, and at varying time points crude extracts of the cells were immunoprecipitated with immunoglobulin G from an antibody to L1210 DHFR as described. Enzyme activity in the crude extract was determined by the methodology described, and the specific radioactivity was determined by dividing the immunoprecipitable counts per minute by the units of DHFR in the extract. The *dashed line* is that obtained after correction for the slope of the cell generation curve (doubling time of 23 hr) and this represents the real turnover rate of the DHFR.

To test this possibility, the half-life of DHFR in cells in the presence and absence of MTX was examined and the results are depicted in Fig. 1. Cells prelabeled with [^{35}S] methionine were grown in nonradioactive chase medium and the decay of the specific radioactivity of the DHFR was measured by an antibody precipitation technique which precipitated only DHFR from the crude extract as described under Methods. The apparent half-life of DHFR was 11.5 hr for cells in the presence or absence of MTX. Thus, MTX had no effect on the turnover of DHFR in these human cells. The *dotted line* is that normalized for the cell generation time showing a real half-life of 23 hr for the DHFR, which is equivalent to the cell generation time. This is considerably shorter than that found in the murine Sarcoma 180 AT/3000 cells (9) and in baby hamster kidney cells (2).

Since the loss of DHFR activity after removal of MTX in Sarcoma 180 AT/3000 cells was irreversible, the reversibility of the DHFR loss in these human KB/6b cells was examined by the readdition of 25 μM MTX, and the results are shown in Fig. 2. The *upper half* of Fig. 2 depicts the growth curve of the cells and indicates no inhibition of cell growth by this concentration of MTX.

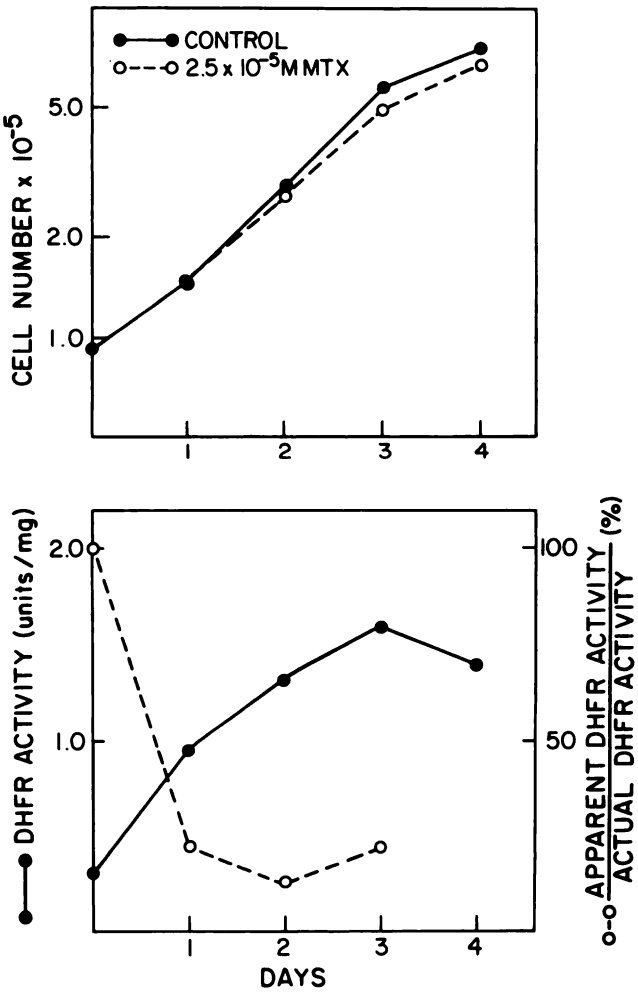

FIG. 2. *Growth curve and DHFR activities of KB/6b cells exposed to 25 μM MTX*

KB/6b cells growing in the absence of MTX were exposed to a selected concentration (25 μM) of MTX. The *upper portion* shows the growth curve for control (●) and MTX-treated (○) cells. The *lower portion* shows the actual DHFR activity (●) in the MTX-treated cells during the same time period. Assays were performed as described in the text, and the ratio of apparent DHFR to actual DHFR (○) is explained in the legend to Fig. 1.

TABLE 2

Effect of methotrexate concentration on induction of dihydrofolate reductase in KB/6b cells

Cells were harvested after 48-hr exposure to MTX, and enzyme was extracted and assayed as described in the text.

MTX concentration	Actual DHFR	Apparent DHFR/ actual DHFR
μM	*units/mg protein*	
0	0.32	100
0.25	0.48	88
0.75	0.73	57
2.5	1.08	22
7.5	1.07	9.6
25	1.05	1.8
75	1.23	.5

The *lower half* shows the time course of the increase of actual DHFR activity. On day 3, the activity found in cells was the same as that before the removal of MTX. The decrease in the ratio of apparent to actual DHFR activity without a change in cell growth suggested an excess amount of DHFR in these cells over that needed for cell growth. The concentration dependence of this increase of DHFR in response to MTX is shown in Table 2. Cells depleted of MTX were re-exposed to MTX concentrations ranging from 0.25 μM to 75 μM for 48 hr. DHFR specific activity increased with increasing MTX concentration up to 2.5 μM. From 2.5 to 25 μM MTX (the selection concentration) the DHFR specific activity was not changed. It should be noted that, at 2.5 μM, about 20% of the DHFR activity is "free." If the increase in DHFR activity is due to the stabilizing effect of MTX on enzyme, it is anticipated that more enzyme activity would be observed at 7.5 μM MTX; this was not the case. These results, together with the results shown in Fig. 1, further support the conclusion that induction of enzyme in these cells is probably not due to enzyme stabilization by MTX. In order to explore the mechanism of this induction process, the role of antimetabolites affecting both RNA and protein synthesis on the increase in DHFR by MTX was examined, and these results are shown in Table 3.

TABLE 3

Action of antimetabolites on the induction of dihydrofolate reductase

MTX concentration was 25 μM, actinomycin D concentration was 10 μg/ml, and Cycloheximide concentration was 20 μg/ml. Cells depleted of MTX were simultaneously exposed to the above drugs for 19 hr, when the cells were harvested and the DHFR was assayed as described in the text.

Antimetabolite	Actual DHFR	Activity changed	% Apparent DHFR/ actual DHFR
	units/mg protein	%	
None	0.32	100	100
MTX	0.55	172	6
Actinomycin D	0.19	59	96
Cycloheximide	0.20	63	86
Actinomycin D + MTX	0.54	169	24
Cycloheximide + MTX	0.32	100	5.9

The 19-hr exposure to MTX resulted in an increase in the actual DHFR from 100% in control cells to 170%. Actinomycin D or cycloheximide alone caused a decrease in actual DHFR, 59% and 63% of uninduced activity, respectively. Since the half-life of DHFR in actinomycin D- or cycloheximide-treated nongrowing cells would be 23 hr, the observed DHFR specific activities at 19 hr after these treatments are consistent with expected values. Cycloheximide added simultaneously with MTX prevented the MTX induction of DHFR activity (58% of MTX alone treated controls), and the activity was the same as in control cells. In contrast, actinomycin D added simultaneously with MTX had no effect on the MTX induction of DHFR activity (98% of cells treated with MTX alone). SDS-polyacrylamide gel protein profiles of these cellular extracts (data not shown) showed an increase in a protein with the same mobility as purified DHFR with MTX and MTX plus actinomycin D treatment.

DISCUSSION

This MTX-resistant human cloned subline of KB cells has an overproduction of DHFR which is similar to the parental cell enzyme. Unlike other "stable" mammalian MTX-resistant cells previously reported (3, 4, 16), these cells have both a stable and unstable component in the increased DHFR, since the activity decreased on removal of MTX from the medium to a stable plateau level which remained 40-fold elevated over that the parental cells. This decrease in DHFR activity was reversible, since the readdition of MTX resulted in an increase of DHFR to predepletion levels. This is in contrast to the observations in the "unstably" amplified Sarcoma 180AT/3000 cell line (9). Furthermore, the increase in DHFR activity was dependent on the concentration of MTX, with enzyme activity increasing with MTX concentration up to 2.5 μM MTX and remaining constant at higher MTX concentrations. This induction of DHFR activity by MTX was not due to stabilization of the enzyme by MTX, since the half-life of the enzyme was the same in the presence and absence of MTX as has previously been shown in another DHFR-overproducing mammalian cell line (9). When we examined the effects of the classic RNA and protein synthesis inhibitors actinomycin D and cycloheximide, respectively, on this MTX-induced modulation, the results showed actinomycin D to have no effect whereas cycloheximide prevented the MTX-associated increase in DHFR activity. DHFR activities with actinomycin D and cycloheximide 19-hr exposures alone (59% and 63% of uninduced activity, respectively) were consistent with those expected for an enzyme half-life of 23 hr. MTX alone caused a 170% increase in DHFR activity which was unaffected by the simultaneous presence of actinomycin D. MTX and cycloheximide resulted in an enzyme activity identical with that of untreated control cells. Since this value is higher than that for cells treated with cycloheximide alone, it seems that cycloheximide is preventing the degradation of the enzyme as well as preventing the MTX induction. These results indicate an effect of MTX on the increase in intracellular DHFR which is not due to stabilization but may be due to a direct or indirect effect of MTX at the translational level. The detailed mechanism(s) of this modulation of

DHFR synthesis by MTX is under current investigation.

An increase in DHFR activity on exposure to MTX was also observed in KB parent cells (data not shown), thus indicating that this phenomenon is not restricted to cells which have adapted to MTX by amplification of the DHFR gene. This rapid increase in DHFR activity on exposure to MTX could have important clinical implications, since the transient increase in DHFR could result in enough enzyme for cellular resistance to the presence of the drug and delayed decrease in DHFR on removal of MTX could be an important factor in the effectiveness of the drug with multidose scheduling. Furthermore, elucidation of the mechanism(s) of this MTX modulation of DHFR activity could lead to improved clinical protocols for MTX treatment by manipulation of the intracellular DHFR activity.

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