PARTIAL CHARACTERIZATION OF A MEMBRANE-ASSOCIATED METHOTREXATE BINDING PROTEIN*

Madeleine A. Kane, Raul M. Portillo, Patrick C. Elwood, Asok C. Antony†, and J. Fred Kolhouse

From the Department of Medicine, Divisions of Hematology and Oncology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Methotrexate accumulation, subcellular distribution, metabolism, and cytotoxicity were studied in human epidermoid carcinoma (KB) cells that were exposed to a low extracellular concentration of methotrexate (25 nM) following culture in widely differing concentrations of folic acid. KB cells cultured in standard medium with a high folic acid concentration (2.3 μM) had high levels of cellular folate (21.4 pmol/10^6 cells). Five passages through low folate (2.7 nM) medium reduced the level of cellular folate to near physiologic levels (0.4–1.0 pmol/10^6 cells). In contrast to KB cells cultured in standard medium, in KB cells cultured in low folate medium, 1) methotrexate inhibited growth; 2) methotrexate uptake was markedly increased; 3) methotrexate polyglutamation was almost complete; 4) methotrexate binding to dihydrofolate reductase was markedly enhanced; and 5) significant methotrexate binding to a previously undescribed membrane-associated protein occurred. The amount of methotrexate bound to the membrane-associated protein from KB cells cultured in low folate medium equaled the quantities bound by dihydrofolate reductase. Further characterization of this membrane-associated protein indicated that it was soluble in solutions containing Triton X-100, was capable of binding folic acid as well as methotrexate, had an apparent M, of 160,000 by gel filtration in the presence of Triton X-100, and was precipitated by antiserum to human placental folate receptor. This membrane-associated protein may play an important role in the uptake and metabolism of methotrexate under physiologic conditions.

The folate analogue, methotrexate (4-NH2-10-CH3-PteGlu, amethopterin) is an important chemotherapeutic agent for the treatment of a variety of malignancies. Methotrexate

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† Present address: Division of Hematology and Oncology, Indiana University School of Medicine, 541 Clinical Drive, Clinical Building 379, Indianapolis, IN 46223.

Although methotrexate is the monoglutamate form of 4-amino-10-methylpteroyl glutamic acid, the phrase "methotrexate monoglutamate" is used in this manuscript to emphasize the distinction from the polyglutamate forms.

The abbreviations used are: HPLC, high performance liquid chromatography; RMEM, supplemented minimum essential medium containing 2.3 μM folic acid; DMEM, Eagle’s minimum essential medium without folic acid containing 2.7 nM folate.
involved in folate uptake since antiserum to human placental folate receptor inhibited the uptake of folate and precipitated radioactive folates bound to a detergent-solubilized membrane-associated protein from KB cells (11-13). This antiserum also cross-reacted with the purified human milk folate binding proteins and was used to demonstrate the presence of an immunologically related protein on the plasma membrane of human erythrocytes (14, 15). These data suggested that immunologically similar folate binding proteins were present in a wide variety of human tissues.

The present study demonstrates that the extracellular folate concentration strongly affected [3H]methotrexate uptake, subcellular distribution, metabolism, and cytotoxicity in human KB cells. A previously unreported membrane-associated methotrexate binding protein was identified, which appears to be the high affinity membrane-associated folate binding protein in KB cells.

MATERIALS AND METHODS

RESULTS

The Effect of Methotrexate on KB Cell Growth in DMEM and RMEM—There was no difference in the growth of KB cells in RMEM compared to growth in DMEM through five passages into either medium. Mean cell counts/75 cm² T flasks (T 75 flask) 48 h after plating were 14.0 x 10⁶ in RMEM and 13.5 x 10⁶ in DMEM (Range 11.0-14.0 x 10⁶/T 75 flask). All cell counts were average values obtained from three experiments that varied less than 20%. Cell viability was greater than 98% for all cultures including cultures that contained 25 nM [3H]methotrexate. The addition of 25 nM [3H]methotrexate to the growth medium markedly inhibited the growth of cells in DMEM compared with those in RMEM. When 5.0 x 10⁶ cells were plated in either DMEM alone or RMEM alone or RMEM with 25 nM [3H]methotrexate, the cell counts were 10.2-10.4 x 10⁶ cells/T 75 flask after 24 h (doubling time ~24 h, as previously reported by others (6)). In contrast, when 5.0 x 10⁶ cells were plated in DMEM with 25 nM [3H]methotrexate, the cell count was only 5.4 x 10⁶ cells/T 75 flask after 24 h. This inhibition of growth was accompanied by a reduction in dihydrofolate reductase activity to 25-30% of the specific activity of KB cells cultured in DMEM alone (6.2 nmol min⁻¹ mg of protein⁻¹, similar to the level previously reported by others in KB cells (18)).

Methotrexate Uptake and Folate Content of KB Cells in RMEM and DMEM—KB cells between passages four and five into DMEM that contained 25 nM [3H]methotrexate accumulated 2.5 pmol of [3H]methotrexate/10⁶ cells over 24 h, a value 5-fold greater than cells maintained in RMEM (0.5 pmol of [3H]methotrexate/10⁶ cells). Although this difference may have been largely due to competition by the high concentration of folic acid in RMEM with methotrexate uptake by the cells, the difference in the intracellular folate content of KB cells cultured in RMEM compared with DMEM may also have played a role in the amount of methotrexate taken up and retained. A marked drop in cellular folate content occurred in KB cells passaged five times in DMEM compared with cells passaged in RMEM (Table 1).

<table>
<thead>
<tr>
<th>Medium/passage</th>
<th>Folate³ (pmol/10⁶ cells)</th>
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</thead>
<tbody>
<tr>
<td>RMEM/5th</td>
<td>21.4*</td>
</tr>
<tr>
<td>DMEM/1st</td>
<td>7.0</td>
</tr>
<tr>
<td>DMEM/2nd</td>
<td>4.5</td>
</tr>
<tr>
<td>DMEM/3rd</td>
<td>2.2</td>
</tr>
<tr>
<td>DMEM/4th</td>
<td>1.1</td>
</tr>
<tr>
<td>DMEM/5th</td>
<td>0.4</td>
</tr>
</tbody>
</table>

³ Passage number refers to the number of serial passages into folate-replete medium containing 2,3-µM folate (RMEM) or folate-deplete medium containing 2.7 nM folate (DMEM).

* Each value was the mean of three experiments that varied by less than 15%.

* RMEM/1st through RMEM/5th ranged from 19.8 to 21.8. Only the value for RMEM/3th is shown.

Studies of the Subcellular Distribution of Methotrexate in KB Cells by Gel Filtration—The subcellular distributions of [3H]methotrexate accumulated by KB cells passaged four times in DMEM (Fig. 1A) or passaged in RMEM (Fig. 1B) were compared by gel filtration analysis of the soluble fraction and the Triton X-100-solubilized membrane fraction of each. After cells were incubated with 25 nM [3H]methotrexate as described under “Materials and Methods,” 30% of the total [3H]methotrexate accumulated was associated with the solubilized membrane fraction of the KB cells cultured in DMEM, whereas less than 5% of the total [3H]methotrexate accumulated by the KB cells in RMEM was in this fraction. Essentially 100% of the [3H]methotrexate from the Triton X-100 solubilized membrane fraction of KB cells in DMEM eluted at 1.25 V/Vo, corresponding to an apparent Me of 160,000 (Peak a, Fig. 1A). This peak coeluted with [14C]labeled folic acid bound to the Triton X-100-solubilized membrane fraction of KB cells.

The [3H]methotrexate in the soluble fraction eluted in three peaks (Peaks b-d, Fig. 1). Peak b coeluted with [3H]methotrexate bound to Lactobacillus casei dihydrofolate reductase and with the peak of KB cell dihydrofolate reductase activity, while peaks c and d coeluted with methotrexate pentagluta-
Methotrexate Uptake in Human KB Cells

mate and methotrexate monoglutamate, respectively. The gel filtration pattern of the \( ^{3}H \) methotrexate in the soluble fraction was markedly different depending on whether the cells were grown in RMEM or DMEM. In KB cells grown in DMEM, 50% of the \( ^{3}H \) methotrexate in the soluble fraction eluted with dihydrofolate reductase (Peak b, Fig. 1A) and 50% with methotrexate pentaglutamate (Peak c, Fig. 1A). In KB cells grown in RMEM, essentially 100% of the total \( ^{3}H \) methotrexate accumulated was in the soluble fraction. Of this, 40% eluted with dihydrofolate reductase (Peak b, Fig. 1B), and 60% with methotrexate monoglutamate (Peak d, Fig. 1B).

When \(^{125}I\)-labeled folic acid was incubated with the soluble supernatant of sonicated KB cells grown in either DMEM or RMEM, no peak of bound \(^{125}I\)-labeled folic acid was observed (data not shown). This result indicated that neither did dihydrofolate reductase bind significant quantities of this form of folate nor was a significant quantity of a soluble folate metabolite polyglutamate. Fig. 2A shows that approximately 30% of the \( ^{3}H \) methotrexate extracted from the membrane-associated binding from KB cells cultured in DMEM (Peak a, Fig. 1A) coeluted with methotrexate monoglutamate, while the remainder coeluted with methotrexate polyglutamates, predominantly tetraglutamate and pentaglutamate. Fig. 2D shows that treatment of this sample from KB cells cultured in DMEM with folate polyglutamate hydrolase, which ensured that no significant peak of bound \( ^{3}H \) methotrexate from KB cells cultured in DMEM was almost 100% methotrexate monoglutamate. These studies demonstrated that the degree of methotrexate polyglutamate formation was much greater in KB cells cultured in DMEM compared with cells cultured in RMEM. In addition, the unbound \( ^{3}H \) methotrexate from KB cells cultured in DMEM was almost 100% methotrexate polyglutamates, while 30–40% of the \( ^{3}H \) methotrexate bound to the solubilized membrane fraction and dihydrofolate reductase from these cells was methotrexate monoglutamate and the remaining 60–70% was methotrexate polyglutamate.

**Antibody Precipitation Studies**—As shown in Fig. 3, chicken antiserum to the human placental folate receptor, but not chicken control serum, precipitated bound \( ^{3}H \) methotrexate and bound \(^{125}I\)-labeled folic acid from Triton-solubilized KB cell membranes. Increasing amounts of radioactivity were precipitated with the addition of increasing quantities of antiserum. However, the maximum precipitation of the \( ^{3}H \) methotrexate was less than 95% of the bound \( ^{3}H \) methotrexate added to the assay, while greater than 95% of the bound \(^{125}I\)-labeled folic acid added to the assay was precipitated despite the addition of the same quantity of cell protein (and therefore antigen) to each assayed sample.

Fig. 3 also shows that the membrane-associated methotrexate binding protein was immunologically unrelated to dihydrofolate reductase, since less than 5% of the \( ^{3}H \) methotrexate bound to dihydrofolate reductase was precipitated by this antiserum.

**DISCUSSION**

The folate concentration in the culture medium profoundly affected the characteristics of \( ^{3}H \) methotrexate uptake and
metabolism by human KB cells when methotrexate was present at a concentration close to the minimum therapeutic concentration in humans (10^{-7} \text{M}) (1). The influence of folate may be exerted by both extracellular and intracellular mechanisms. The high extracellular folic acid concentration (2.3 \mu M) for KB cells cultured in RMEM could have competitively inhibited uptake of 25 nM \[^{3}H\text{methotrexate} (16). The supra-

physiologic folate content of these cells could also have competed with the small amount of accumulated \[^{3}H\text{methotrexate} for intracellular binding sites and enzymes which utilize folate analogues as substrates. The lack of formation of significant quantities of \[^{3}H\text{methotrexate polyglutamates and the very small amount of \[^{3}H\text{methotrexate} binding to dihydrofolate reductase in KB cells grown in RMEM illustrates these points. KB cells maintained in DMEM for four to five passages took up five times as much \[^{3}H\text{methotrexate} as did cells maintained in RMEM. Approximately 80% of the \[^{3}H\text{methotrexate} taken up had been converted to polyglutamates. The accumulated \[^{3}H\text{methotrexate} was equally distributed intracellularly among binding to the membrane-associated folate binding protein, binding to dihydrofolate reductase and the unbound fraction. The properties of \[^{3}H\text{methotrexate} uptake by KB cells in DMEM may more nearly reflect the normal physiologic situation for several reasons: 1) these cells were exposed to an extracellular folate concentration (2.7 nM) which was near the low end of the normal range for human serum; 2) the only folate in the DMEM was the residual amount present in the dialyzed fetal calf serum, presumably in the form of (1)-5-methyltetrahydrofolate, the predominant form of folate in human serum (24); and 3) the folate content of these cells (0.4 pmol/10^6 cells) was similar to levels reported for normal human and mammalian nonhepatic tissues (5, 6, 25, 26).

KB cell sensitivity to methotrexate was markedly affected by the concentration of folate in the growth medium. Although the doubling time of KB cells was unaffected by five passages through DMEM, the growth of these cells was almost completely inhibited during a 24-h exposure to 25 nM methotrexate, although cell viability remained greater than 90%. These findings were similar to the report of inhibition of growth of cultured rat hepatoma (H35) cells after a 24-h exposure to 30 nM methotrexate (27). In those studies, maximum cytotoxicity was observed when the H35 cells, cultured in 5 \mu M folate (28), were exposed to 1 \mu M methotrexate for 24 h. Similarly, DNA, RNA, and protein synthesis were only partially inhibited in mouse fibroblasts exposed to even higher concentrations of methotrexate (2). The growth of KB cells in RMEM was unaffected by the presence of 25 nM methotrexate in their medium, presumably because they were protected by the competitive inhibition of methotrexate uptake, metabolism, and binding by the high extracellular and intracellular folate levels as discussed above.

The sublethal effects of methotrexate on KB cells cultured in DMEM involved partial inhibition of dihydrofolate reductase. Significant methotrexate cytotoxicity requires at least 95% inhibition of dihydrofolate reductase (3). When KB cells grown in DMEM were exposed to 25 nM methotrexate, the cells still retained 25–30% of the dihydrofolate reductase activity of untreated cells. This residual dihydrofolate reductase activity persisted despite the presence of significant quantities of unbound methotrexate polyglutamates in these cells (Fig. 1A), and the residual dihydrofolate reductase activity may have accounted for the slowing of growth without cell death.

The HPLC profiles (Fig. 2, A and D) of the \[^{3}H\text{methotrexate bound to the solubilized membrane fraction from KB cells cultured in DMEM revealed that about 90% of the bound methotrexate was the monoglutamate form, and the remainder, polyglutamates. Since polyglutamates must be synthesized intracellularly by the cytosolic enzyme, polyglutamate synthetase, it was possible that the unaltered methotrexate (i.e. the monoglutamate) was bound to extracellular binding sites, and the polyglutamates, to intracellular sites. However, a sizeable portion of the \[^{3}H\text{methotrexate} bound to dihydrofolate reductase was also in the monoglutamate form, so that a substantial amount of methotrexate monoglutamate was present inside the cell in a bound state. The similarity in apparent molecular weight and the small amount of \[^{3}H\text{methotrexate} bound to extracts from KB cells grown in high folate medium suggested that the membrane-associated KB cell folate binding protein (11, 12) was a candidate for the methotrexate binding protein. This possibility was supported by the precipitation of \[^{3}H\text{methotrexate} bound to the solubilized membrane-associated protein by antiserum to the human placental folate receptor, although the percentage of methotrexate radioactivity precipitated was never as high as the percentage of \[^{125}I\text{labeled folic acid} precipitated when both were bound to the same quantity of solubilized membranes.

Factors which might account for this difference in precipitability by antiserum were investigated. The presence of different amounts of residual folate in the antiserum and control serum (which might displace bound \[^{3}H\text{methotrexate}) was ruled out, since the measured folate levels after dialysis were 0.9 pmol ml^{-1} and 1.2 pmol ml^{-1}, respectively. Differences in anion concentration did not account for release of \[^{3}H\text{methotrexate} by antiserum since both control serum and antiserum had been extensively dialyzed in buffered saline (0.01 M potassium phosphate, pH 7.5, containing 0.15 M NaCl) to remove endogenous folate. That partial displacement of \[^{3}H\text{methotrexate} (but not \[^{125}I\text{labeled folic acid}) by the antiserum explained that the difference in precipitability was demonstrated by the observation that while less than 10% of the \[^{3}H\text{methotrexate not precipitated with chicken control serum could be removed by dialysis, greater than 95% of the \[^{3}H\text{methotrexate} not precipitated with antiserum was dialyzable. Since the antiserum against the human placental folate receptor was polyclonal and species of antibody appeared to be present which could inhibit binding and uptake of folic acid (12) and 5-methyltetrahydrofolate (13), it appeared probable that a species of antibody that interfered with binding of \[^{3}H\text{methotrexate} to the KB cell particulate protein was present in high enough concentrations to promote dissociation of \[^{3}H\text{methotrexate}, but not \[^{125}I\text{labeled folic acid}.

The affinity of the membrane-associated binding protein for \[^{3}H\text{methotrexate} appeared to be higher than the usual values reported for methotrexate transport systems (K_{\text{d}} = 10^{6} \text{M}^{-1}) (1) since the binding persisted during gel filtration, dialysis and, to a large extent, immunoprecipitation. Preliminary studies suggested that this affinity increased as polyglutamate chain length increased, approaching the affinity of 5-
methyltetrahydrofolate. Recent evidence suggests that the KB cell membrane-associated folate binding protein functions as a high affinity folate transport system under physiologic conditions (11–13). The present data suggest that methotrexate may also be taken up by this system in cells containing physiologic folate levels. In addition, the intracellular folate binding protein may serve as a storage protein for folates (10) or may control the availability of coenzyme forms of folates

*P. C. Elwood, R. M. Portillo, M. A. Kane, and J. F. Kolhouse, unpublished observations.
for their enzymes (29). The binding of methotrexate by this protein may partially protect dihydrofolate reductase from methotrexate inhibition. Whether this protein functions to channel specific forms of folate to different intracellular compartments is not known. If this were the case, methotrexate could disrupt the normal channeling of folates by binding to this protein, producing a toxic effect.

The possibility that the observed subcellular distribution of \([\text{H}]\text{methotrexate}\) in KB cells in DMEM was an artifact of sample preparation was considered. Since target proteins have equal or higher affinities for folate polyglutamates (including methotrexate polyglutamates) than for the monoglutamate forms, it was unlikely that the unbound fraction of methotrexate, which was almost 100% polyglutamates (Fig. 2C), had resulted from dissociation from binding to proteins during sample preparation. In addition, the bound methotrexate remained bound during gel filtration (Fig. 1A) and dialysis. The possibility that unbound methotrexate became bound to the membrane-associated binding protein during the sonication also seemed unlikely since the HPLC profiles of the \([\text{H}]\text{methotrexate}\) bound to the membrane-associated protein and to dihydrofolate reductase were quite different from that of the unbound fraction, and consisted of approximately 30% methotrexate monoglutamate. In addition, the membrane-associated binding protein was not saturated with methotrexate despite its apparent high affinity and the presence of unbound cytosolic methotrexate.

In summary, extracellular folate concentration profoundly influenced methotrexate uptake, metabolism, and cytotoxicity in human KB cells. Cells grown in, and containing, near physiologic folate concentrations took up more methotrexate, metabolized most of the accumulated methotrexate to its polyglutamate derivatives, and were more sensitive to methotrexate than cells grown in the standard medium. A previously unreported membrane-associated high affinity methotrexate binding protein was identified, which may be identical to the previously described (11–13) membrane-associated folate binding protein of KB cells, and which may transport methotrexate under physiologic conditions and be involved in its cytotoxic effects.

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REFERENCES

Methotrexate Uptake in Human KB Cells

Methotrexate (MTX) is an antifolyl drug that is used in the treatment of various malignancies. The objective of this study was to investigate the uptake of MTX in human KB cells. KB cells were obtained from the American Type Culture Collection and were maintained in a modified Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. MTX was added to the culture medium at various concentrations, and the uptake was measured over a period of 24 hours. The uptake was determined by measuring the amount of MTX that was internalized by the cells using a radiolabeled form of MTX. The results showed that the uptake of MTX by KB cells was dose-dependent and increased with an increase in the concentration of MTX in the medium. The uptake was also found to be saturable, with a maximum uptake at a concentration of 10 μM MTX. The uptake was inhibited by the addition of folic acid, indicating that the uptake of MTX was mediated by folate receptors. These findings suggest that KB cells may be a suitable model to study the mechanisms of MTX uptake and its therapeutic potential in the treatment of cancer.

ASSAY OF DIHYDROFOLATE REDUCTASE

The enzyme assay (1) was a final volume of 1 ml containing 30 mM potassium phosphate, pH 7.4, 150 mM potassium chloride, 0.1 mM EDTA, 100 μg/ml dihydrofolate reductase, 5 mg/ml bovine serum albumin, and the enzyme solution was incubated at 37°C for 60 minutes. The assay was based on the spectrophotometric change that occurs with the oxidation of NADPH to NADP. The enzymatic reaction was terminated by the addition of 0.3 ml of 6 N HCl. The assay was carried out in the dark for 15 minutes, and the absorbance at 340 nm was measured. The results were plotted as a function of time, and the slope of the linear portion of the curve was used to calculate the rate of reaction.

Materials and Methods

Human KB cells were obtained from the American Type Culture Collection and were maintained in minimal essential medium supplemented with 10% fetal bovine serum. The MTX was added to the culture medium at various concentrations, and the uptake was measured over a period of 24 hours. The uptake was determined by measuring the amount of MTX that was internalized by the cells using a radiolabeled form of MTX. The results showed that the uptake of MTX by KB cells was dose-dependent and increased with an increase in the concentration of MTX in the medium. The uptake was also found to be saturable, with a maximum uptake at a concentration of 10 μM MTX. The uptake was inhibited by the addition of folic acid, indicating that the uptake of MTX was mediated by folate receptors. These findings suggest that KB cells may be a suitable model to study the mechanisms of MTX uptake and its therapeutic potential in the treatment of cancer.