PREVENTION BY THYMIDINE AGAINST TOXICITY AND GLUCOSE UPTAKE INHIBITION OF METHOTREXATE ON CULTURED EHRLICH ASCITES TUMOUR CELLS

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SUMMARY

Methotrexate (MTX) arrested cell growth and inhibited 2-deoxy-D-glucose uptake of Ehrlich ascites tumour cells in vitro. The changes of glucose concentrations in the culture media did not affect the degree of suppression of cell growth by MTX. Thymidine (dThd) protected against the toxic effect of MTX on the cells. It also prevented the inhibition effect on glucose uptake of the cells by MTX. MTX significantly suppressed the maximal uptake rate of glucose (V_{max}) while addition of dThd alleviated the suppression. The half-saturation constant of the uptake (K_m) remained constant.

INTRODUCTION

Methotrexate (MTX) is a commonly used antimetabolite in cancer chemotherapy. However, its mode of action on tumour cell is not fully clear. It is generally believed that MTX arrests the growth of tumour cells by inhibiting nucleotide synthesis and the induction of a "purineless" state in these cells (Hryniuk, 1972). Thus, it is reasonable to observe that the growth inhibitory effects of MTX on tumour cell lines are decreased in the presence of exogenous purines (Hakala et al., 1961; Borsa & Whitmore, 1969a; Hryniuk & Bertino, 1971; Kaminska, 1979). It is a poorly understood fact

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that exogenous thymidine (dThd) can also partially protect against cytotoxic effect of MTX on some tumour cell lines, for examples in mouse L cells (Borsa & Whitmore, 1969b; Moran et al., 1979) and human lymphoblastic leukemia cells (Moran et al., 1979). The partial protective effects by dThd against the cytotoxicity of MTX on tumour cells have been interpreted to mean that inhibition of dihydrofolate reductase is insufficient to explain the anti-tumour effects of MTX (Hryniuk, 1972; McBurney & Whitmore, 1975; Rosenfeld, 1975). Recently, Moran et al. (1979) proposed that the protection against the cytotoxic effects of MTX by exogenous purine and dThd have a common biochemical mechanism namely, inhibition of the de novo synthesis of thymidylate through feedback inhibition by anabolites on ribonucleotide reductase and deoxycytidylate deaminase.

In the present communication, we study the effect of dThd on the cytotoxicity of MTX on Ehrlich ascites tumour (EAT) cells in vitro. EAT cells depend primarily on glycolysis for the provision of energy (Live & Kaminskas, 1975). We (Chan et al., 1983) have shown that MTX arrests growth and inhibits glucose uptake of EAT cells in vivo. Kaminskas (1979) also showed that MTX inhibits glucose uptake and glycolysis rate of EAT cells in vitro. The effect of dThd on the glucose uptake rate of MTX treated EAT cells was therefore also examined.

METHODS

Cell Cultivation

Mouse Ehrlich ascites tumour (EAT) cells were cultured in RPMI 1640 (KC Biological) supplemented with 10% heat-inactivated fetal calf serum (Gibco) 2 mM L-glutamine, 30 mM glucose, 50 units/ml penicillin and 100 μg/ml streptomycin (Gibco). The medium was buffered with 25 mM HEPES (Sigma) and 25 mM $NaHCO_3$. For testing the effect of changes of glucose concentrations

on the cytotoxicity of MTX on EAT cells, different amounts of glucose were supplemented to the cultures.

Drug Treatment

MTX (Lederle) was reconstituted with distilled water and sterilized by passage through millipore filter. The solution was diluted with culture medium to desired concentration before use.

dThd (Sigma) was dissolved in cultured medium before use. Pilot studies have been coducted to obtain optimal dose and treatment schedule of dThd for the maximal protection against toxicity of MTX on cultured EAT cells. In our experiments, the cell concentration was 5×10^5 cell/ml, MTX concentrations ranged from 0.02 - 20 μM and the dThd concentration was 40 μM. All concentrations fall in the range of those used by Moran et al. (1979) in the study of effect of dThd on MTX treated L cells. We also found that preincubation of EAT cells with dThd 2 h prior to MTX addition facilitated maximal protection of dThd against cytotoxicity. This incubation schedule was therefore followed throughout our experiments.

EAT cell count was determined with a hemocytometer. The cell viability was assayed by trypan blue exclusion.

Uptake of 2-deoxy-D-glucose

2-Deoxy-D-glucose uptake of EAT cells was measured as previously described (Leung et al., 1984; Fung et al., 1985). Cultured EAT cells were washed with phosphate buffered saline, pH 7.4 (PBS). Cell suspension (2×10^7/ml in PBS) was equilibrated to 37°C. At 0 s, 0.2 ml of suspension was mixed with 0.2 ml of prewarmed 2-deoxy-D-[^{3}H]glucose (1 μCi/mil) to give a final concentration of 0.25 - 4.0 mM. Reaction was stopped after 6 s by transferring 0.2 ml of the mixture to 1 ml of ice-cold PBS supplemented with 40 mM 2-deoxy-D-glucose. Cells were collected by centrifugation at 15,000 x g for 10 s in an Eppendorf 5414 microcentrifuge. The supernate was

removed and the cells washed with 1 ml of the same buffer. Cell lysis was accomplished by the addition of 0.2 ml 0.1% Triton X-100. One millilitre of Triton X-toluene scintillanat (Patterson & Greene, 1965) was added and the radioactivity counted in a Beckman LS7000 liquid scintillation counter. Nonspecific diffusion was corrected by subtracting the uptake in the presence of 10 μM cytochalasin B. The kinetic parameters V_{max} and K_m, representing respectively the maximal uptake and apparent half-saturation constant for the specific transport process, were determined by double-reciprocal plots.

RESULTS

Figure 1A shows the inhibition of proliferation of EAT cells by MTX. MTX suppressed cell growth in a dose dependent manner. However, similar suppresion was observed in 24 h or 48 h cultures in the presence of same amount of MTX. Figure 1A also shows that the inhibition of proliferation of EAT cells by MTX was decreased by dThd. The effects of protection of dThd on growth were more pronounced in cells treated with higher concentration of MTX or with longer period.

Concerning the cell viability, EAT cells remained about 90% viable at different MTX doses in cultures with 24 h incubation (Figure 1B). However, the viability dropped drastically when the cells were treated with MTX at 0.02 μM or higher concentreations for 48 h. These drops in viability could be protected in the presence of 40 μM dThd (Figure 1B).

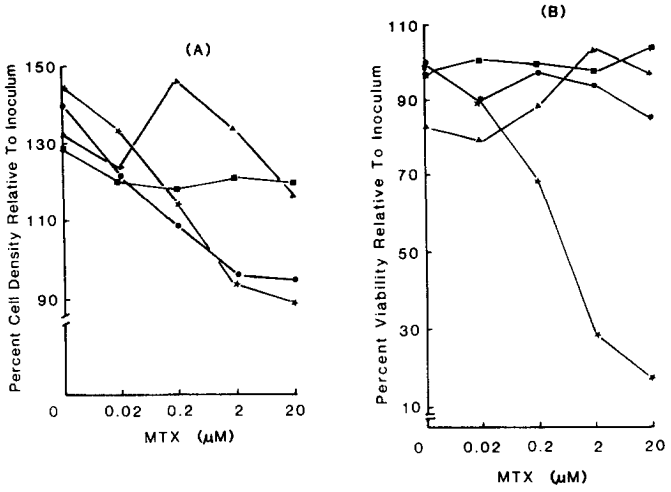

Figure 1.A. Effect of dThd on the proliferation of MTX treated EAT cells in vitro

EAT cells (2 x 10^5 cells/ml) were incubated with 40 μM dThd in culture medium or suspended in culture medium for 2 h. MTX were added at indicated concentrations and incubated for further 24 h or 48 h. Cell counts were determined by a hemocytometer. (●—●), cells incubated in MTX for 24 h; (✱—✱), cells incubated in MTX for 48 h; (■—■), cells incubated in MTX and dThd for 24 h; (▲—▲), cells incubated in MTX and dThd for 48 h. Cell density after incubation was expressed as percentage relative to inoculum. Values were average of results of two experiments.

B. Effect of dThd on the viability of MTX treated EAT cells in vitro

Same experimental protocol as in 1A. Cell viability after incubation was assayed by trypan blue exclusion and expressed as percentage relative to inculum. Values were average of results of two experiments.

Table 1. Effect of dThd on the 2-deoxyglucose uptake of MTX treated EAT cells in vitro.

Treatment	V_{max} (nmol/10^6 cells/min)	K_m (mM)
Medium (control)	18.8 ± 0.8	0.97 ± 0.06
MTX	15.9 ± 0.8†	0.91 ± 0.08
MTX + dThd	20.3 ± 1.1	1.10 ± 0.07
dThd	21.2 ± 3.0	1.29 ± 0.24

Note: EAT cells (2 x 10^5 cells/ml) were incubated with 40 μM dThd in culture medium or suspended in culture medium for 2 h. For the MTX-treated groups, 2 μM MTX were added. The cells were further incubated for 24 h. Cells were washed with phosphate-buffered saline, pH 7.4 and the 2-deoxyglucose uptake was determined as described in METHODS. Values are presented as mean ± SEM. The level of significance was evaluated by Student's t-test. †, $p<0.05$ when compared with control.

Table 1 shows the changes of the uptake of 2-deoxyglucose of cultured EAT cells under different drug treatments. Incubation of cells with 2 μM MTX for 24 h reduced the V_{max} of 2-deoxyglucose uptake process significantly ($p<0.05$) by about 15% when compared with that of untreated cells. When the cells were incubated with MTX in the presence of 40 μM dThd, no reduction of V_{max} of uptake process was observed as compared with that of control. dThd itself caused no change in V_{max} value. In both drug treatments, the apparent K_m values for glucose uptake were unaffected when compared with that of untreated cells.

In table 1, MTX has been shown to reduce glucose uptake in cultured EAT cells during the course of tumour growth suppression. It seems reasonable to test whether changes of glucose content in the culture media could affect the cytotoxic effect of MTX on EAT cells. Table 2 shows that EAT cells grown in RPMI - 1640 culture media supplemented with 30, 35, 40 and 50 mM glucose were suppressed to similar extent by 2 μM MTX after 24 h incubation.

Table 2. Effect of different concentrations of glucose in the culture media on the cytotoxicity of MTX on EAT cells in vitro.

Glucose concentration (mM)	Cell numbers ($X10^{-5}$)	
	Control	MTX-treated
30	5.8 ± 0.2	2.7 ± 0.4
35	6.1 ± 0.5	2.7 ± 0.2
40	6.2 ± 0.4	2.8 ± 0.2
50	6.7 ± 0.3	2.9 ± 0.3

Note: EAT cells (2 x 10^5 cells/ml) were incubated in RPMI-1640 culture media supplemented with different concentrations of glucose as indicated. In the MTX-treated groups, 2 μM MTX were added. Cell densities after 24 h incubation were recorded as described in METHODS. Values are presented as mean ± SEM.

DISCUSSION

The present study shows that the cytotoxic effect of MTX in cultured EAT cells can be prevented by exogenous dThd (Figure 1). The partial protection by dThd against MTX toxicity have also been reported in mouse L cells (Borsa & Whitmore, 1969b; Moran et al., 1979) and human lymphoblastic leukemia cells (Moran et al., 1979). We further showed that the glucose uptake inhibition of MTX on EAT cells can also be prevented by dThd. Table 1 showed that MTX significantly suppressed the V_{max} of glucose uptake process when compared with that of control while addition of dThd alleviated the suppression. In both MTX treated, MTX and dThd treated and untreated EAT cells, the values of apparent K_m values remained relatively unchanged. Qualitative change in the affinity of glucose and its transport component in the cells after treatments need not be invoked.

In the consideration of the primary biochemical mode of actions of MTX on tumour cells, little concern has been paid in literatures on the

inhibitory effect of MTX on energy metabolism of tumour cells. Glucose is a major metabolic substrate of many tumour cells. The transport of glucose across the plasma membranes of a variety of tumour cells including Novikoff rat hepatoma cells (Graff et al., 1981) and cultured EAT cells (Cuppoletti et al., 1981; Chan et al., 1983) have been demonstrated to be carrier mediated. It is reasonably certain from the present data (table 1) that MTX induces decreases in carrier-meidated glucose transport in EAT cells in vitro, thereby suppressing tumour growth. The cytotoxic effect of MTX on EAT cells in vitro seems to be independent on the exogenous glucose concentrations in the culture media (table 2). However, the actual mechanism of MTX on the regulation of glucose transport of EAT cells is not at all clear. It remains to be ascertained, for example, whether MTX also acts on the simple diffusion rate of glucose of EAT cells. Glucose diffuses across the cell membrane very slowly in most cell types and increases of exogenous glucose concentrations in cultured cells might enhance the glucose diffusion rate (Elbrink & Bihler, 1975) but not the carrier-mediated transport rate (Amos et al., 1977; Fung et al., 1986). Our results in table 2 showing that increases of glucose concentrations of culture media exhibit no effect on MTX cytotoxicity on EAT cells might suggest that increases of simple diffusion of glucose in the EAT cells exert no effect on MTX cytotoxicity. We have also shown that dThd protected the cytotoxic effect of MTX on EAT cells (Figure 1). It also prevented the glucose uptake inhibition of MTX in the tumour cells (Table 1). Though at present it is difficult to understand the mechanism of change of glucose uptake in MTX treated cells in the presence of dThd, the paralleled changes of cell growth and glucose uptake in MTX treated and MTX and dThd treated EAT cells would mean the glucose metabolism is at least one of the important factors contributing to the mode of actions of MTX on EAT cells in vitro.

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