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DIFFERENTIAL TOXICITY OF CARRIER-BOUND METHOTREXATE TOWARD HUMAN LYMPHOCYTES, MARROW AND TUMOR CELLS

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Abstract—Methotrexate that was covalently linked to poly-L-lysine (mol. wt 3,000 and 60,000) (MTX–PLL 3K and 60K) was more inhibitory to the growth of five cell lines from human solid tumors (IC_{50} 5–10 × 10^{-8} M and 1–2.6 × 10^{-8} M respectively) than to the growth of five lines of human lymphocytes (IC_{50} 5–8 × 10^{-7} M and 2–5 × 10^{-7} M). In contrast, both methotrexate that was covalently linked to human serum albumin (MTX–HSA), and the free drug, were equally toxic to the two classes of cells, with IC_{50} of 3–15 × 10^{-7} M and 2–7 × 10^{-8} M, respectively, for the cell types. Uptake studies showed that, whereas MTX and MTX–HSA were transported equally well into WI-L2 lymphocytes, human bone marrow cells, and an astrocytoma tumor line, uptake of MTX–PLL by the astrocytoma cells at 37° was three to four times greater than uptake by WI-L2 lymphocytes or marrow cells. [^{3}H]Deoxyuridine ([^{3}H]-dUrd) incorporation studies indicated that low concentrations of MTX–PLL 60K (5 × 10^{-7} M) resulted in inhibition of the target enzyme dihydrofolate reductase (DHFR) in the astrocytoma cells, but no inhibition of DHFR occurred in WI-L2 lymphocytes or marrow cells until concentrations were reached where the carrier itself became toxic (5 × 10^{-6} M). Two inhibitors of the lysosomal enzymes, chloroquine and lupeptin, were able to reverse the toxicity of MTX–PLL 60K against the astrocytoma tumor cell line, increasing its IC_{50} from 2 × 10^{-8} to 2 × 10^{-7} M. Both lysosomal inhibitors had no effect on the toxicity of MTX–PLL 60K against the WI-L2 lymphocytes or of MTX or MTX–HSA against either cell type, indicating that the increased toxicity of MTX–PLL 60K against the tumor cells was due, in part, to the ability of the lysosomes of these cells to convert MTX–PLL 60K either to the free drug or to a derivative that was effective in inactivating DHFR. These results suggest that comparable differential toxicity between marrow and tumor cells might also be achieved *in vivo* if MTX–PLL is infused over long periods at a rate that would maintain a constant serum concentration sufficient to kill tumor cells without affecting bone marrow cells.

Clinical cancer chemotherapy is hindered by the lack of selective toxicity of most anti-cancer agents for tumor cells relative to normal cells. In particular, toxicity to the rapidly dividing marrow and intestinal mucosal cells is often the limiting factor in continued and successful therapy. Although some selectivity can be achieved by utilizing the increased metabolic activity in many tumor cells, this difference is not large enough for it to be generally satisfactory. A major problem is that most anti-cancer drugs are small molecules that are transported into cells through carrier-mediated mechanisms that are not substantially different in normal and tumor cells, and they inhibit cell growth by interfering with biochemical pathways common to both types of cells.

It may be possible, however, to utilize carrier molecules attached to drugs to achieve more selectivity than can be attained by the parent compounds. For example, it has been shown that the transport of proteins into cells, which takes place through the process of pinocytosis [1, 2], occurs more readily in some types of tumor cells than in bone marrow cells *in vivo* [3] and that, when radioactively labeled proteins were injected into tumor-bearing rats *in vivo*, the amount of label found in the tumors was significantly greater than that in other tissues [4–6]. These results indicate the existence of some selectivity in the protein uptake of normal and tumor cells and suggest that, if proteins were used as drug carriers, they might be preferentially taken up by tumor cells rather than by the vulnerable marrow cells. In addition to possible differential uptake, increased lysosomal activity has been reported in many tumor cells [7]. Because the breakdown of proteins and other macromolecules is believed to occur in the lysosomes [8, 9] and because this breakdown is necessary to liberate the drug before it can exert its cytotoxic effects [10, 11], an additional avenue of selectivity might be achieved by the use of drugs attached to carriers.

In this study we have determined whether differential toxicity to different cell types can be achieved *in vitro* when MTX is covalently linked to serum albumin or poly-L-lysine, using five human tumor cell lines established from patients with non-lymphomatous solid tumors, five EBV transformed human lymphocyte lines, and normal human marrow mononuclear cells. MTX, coupled to either serum albumin or poly-L-lysine, has been shown previously to be effective *in vivo* against both MTX sensitive and resistant mouse tumors [12–14].

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MATERIALS AND METHODS

Chemicals. The following were obtained from commercial sources: MTX,* sodium salt, Lederle Laboratories, Pearl River, NY; [3′,5′,7-^{3}H]-MTX (250 mCi or 20 Ci/mmole), Amersham Radiochemicals, Chicago, IL; [6-^{3}H]-deoxyuridine (24.2 Ci/mmole), New England Nuclear Corp., Boston, MA; human serum albumin, poly-L-lysine (mol. wt 3,000 and 60,000), chloroquine, and lupeptin, Sigma Chemical Co., St. Louis, MO; and phosphate-buffered saline tablets, Oxoid Ltd., England.

MTX–PLL 3K, MTX–PLL 60K, [^{3}H]-MTX–PLL 3K (6.5 mCi/mmole), and [^{3}H]-MTX–PLL 60K (3.5 mCi/mmole) were synthesized using the method previously described for synthesizing MTX–BSA [15], except that the products were separated from free MTX on a Sephadex G-25 column (Pharmacia Chemicals, Piscataway, NJ) and eluted with phosphate-buffered saline, pH 7.5 (PBS). Several different MTX–PLL preparations, ranging between 83.3 and 113.4 mg/g PLL were used in the experiments reported here. MTX–HSA and [^{3}H]-MTX–HSA (14.7 mCi/mmole) were synthesized as described previously [15]. Chromatography of [^{3}H]-MTX–HSA and the [^{3}H]-MTX–PLL derivatives on Whatman No. 1 paper (MeOH–NH_3–H_2O; 7:1:2) showed that the preparations contained less than 0.3% free MTX.†

Cell lines. Cell lines were obtained from the following sources at the University of California School of Medicine, La Jolla, CA: WI-L2 [16] and CEM [17], Dr. J. Seegmiller; SB [18], HSB [18] and 8402 [19], Dr. I. Royston, T24A astrocytoma, T242 melanoma and T24B bladder carcinoma, Dr. H. Masui (H. Masui and T. White, manuscript in preparation); and hepatocarcinoma and osteosarcoma, Dr. A. Yu. The lymphocytic lines were originally isolated from patients with lymphocytic leukemia. Morphological and karyotype studies indicated that lines HSB, CEM and 8402 were probably derived from malignant T lymphocytes, whereas the WI-L2 and SB lines were derived from normal cells that were present in the leukemic blood. Only line SB carried the EBV (Epstein–Barr virus) genome. Tumor cells originated from patients with non-lymphomatous solid tumors, and the lines were established in culture from a biopsy of the tumor xenograft in nude mice. Human bone marrow mononuclear cells were obtained from patients undergoing diagnostic evaluation or treatment unrelated to these studies. The mononuclear cells were separated from erythrocytes and polynuclear cells on a Ficoll–Hypaque gradient [20].

In vitro *cell growth experiments.* All cells were grown in 24-welled Linbro plates (Flow Labs, Inglewood, CA) in Autopow media (Flow Labs) supplemented with 10% fetal calf serum (Irvine Scientific Co., Los Angeles, CA) and 1% antibiotic-antimycotic solution (Gibco Scientific Co., Grand Island, NY), starting at a concentration of 0.2×10^5 cells/ml. Drugs were added to the lymphocytes within 0.5 hr of plating; tumor lines were allowed 15–20 hr to attach to the plates before addition of drug; no significant growth occurred during this time. The cells were counted when the untreated control cells had undergone approximately three doublings (or reached a concentration of 1.2 to 1.5×10^5 cells/ml), using the model ZBI Coulter counter. In experiments where the drugs were removed after 2 or 24 hr of drug incubation, cells were washed once with PBS and resuspended in fresh medium. Chloroquine or lupeptin was added to the cells at the same time as MTX and its derivatives. The average time needed for cells to reach 1.2 to 1.5×10^5 cells/ml from an initial concentration of 0.2×10^5 cells/ml were: WI-L2 and CEM, 2 days; all other cell lines, 3–4 days.

[^{3}H]-*dUrd incorporation experiments.* WI-L2 and human mononuclear marrow cells (2–4 $\times 10^5$ cells/ml) were pretreated with increasing concentrations of MTX and its derivatives for 4 hr at 37° in the same media used for growth experiments to establish the concentration ranges over which per cent inhibition of [^{3}H]-dUrd incorporation into DNA relative to control untreated cells was linear (5–90 per cent). In experiments where chloroquine was used, it was added to the cells simultaneously with drugs. The drug containing medium was then removed by centrifugation and the cells were suspended in fresh medium (without fetal calf serum). [^{3}H]-dUrd (2 μCi/ml) was then added to the cells; after 15 min at 37° the incorporation was stopped by addition of cold PBS. The cells were then centrifuged, washed and counted. Aliquots of $1–2 \times 10^5$ cells were precipitated with 10% trichloroacetic acid and the precipitate was collected on GF/C filters (Whatman), dried, suspended in Omnifluor scintillant, and counted in a Beckman LS-100C counter, as described previously [21]. [^{3}H]-dUrd incorporation into DNA in all cell lines was linear during the 15-min interval. T24A astrocytoma cells were plated in 6-welled Linbro plates 24 hr before the start of the experiment. The same procedure described above was followed, except that the media were removed from the cells by aspiration and the cells were removed from plates by gentle aspiration with 5 ml PBS.

Uptake of [^{3}H]-*MTX,* [^{3}H]-*MTX–HSA and* [^{3}H]-*MTX–PLL 3K and 60K by WI-L2, T24A and human marrow cells.* Cells (0.5 to 2×10^6/ml) were incubated with 5×10^{-7} M [^{3}H]-MTX (227 mCi/mmole, purified by paper chromatography), 5×10^{-6} M [^{3}H]-MTX–HSA (14.7 mCi/mmole), 5×10^{-7} M [^{3}H]-MTX–PLL 60K (3.5 mCi/mmole) or 5×10^{-7} M [^{3}H]-MTX–PLL 3K (6.5 mCi/mmole) for 0.5, 1 and 2 hr at 37° or 0° in the same media used for growth experiments. The cells were then washed with cold PBS to remove external radioactivity and resuspended in 1 ml PBS; an aliquot was counted in the Coulter counter and the rest was mixed with 10 ml Aquasol (New England Nuclear Corp.) and counted in a Beckman LS-100C scintillation counter. In some experiments the cells were lysed by freezing and thawing and the membrane

* Abbreviations: MTX, methotrexate; MTX–HSA, MTX–human serum albumin; MTX–PLL 3K and 60K, MTX–poly-L-lysine (mol. wt 3,000 and 60,000); [^{3}H]-dUrd, [^{3}H]-deoxyuridine; DHFR, dihydrofolate reductase; PBS, phosphate-buffered saline; and IC_{50}-concentration of drug needed to reduce rate of growth of cells by 50 per cent.

† All concentrations reported for MTX-HSA and MTX-PLL refer to concentration of bound MTX.

Table 1. IC_{50} Values of MTX, MTX-HSA, and MTX-PLL 3K and 60K against five human lymphocyte and five human tumor lines and the ratio (IC_{50} MTX–carrier/IC_{50} free MTX) for these lines*

Type	MTX ($\times 10^{-7}$ M)	MTX–HSA ($\times 10^{-7}$ M)	R	MTX–PLL 3K ($\times 10^{-7}$ M)	R	MTX–PLL 60K ($\times 10^{-7}$ M)	R	PLL 60K (μg/ml)
Lymphocytes								
WI-L2 B	0.28 ± 0.09	7.3 ± 1.7	26	5.0 ± 1.0	18	3.3 ± 1.3	12	25†
SB B	0.71 ± 0.12	16 ± 6.5	22	6.3 ± 2.3	9	4.8 ± 2.2	7	25†
CEM T	0.23 ± 0.11	10 ± 7.5	43	4.8 ± 1.5	21	2.9 ± 2.2	13	25†
HSB T	0.40 ± 0.29	14 ± 11.0	35	5.0 ± 4.7	12	2.5 ± 1.9	6	25†
8402 T	0.18 ± 0.02	3.2 ± 0.1	18	7.0 ± 2.0	39	3.0 ± 0.2	17	ND‡
Tumor								
T24A (astrocytoma)	0.21 ± 0.12	3.4 ± 1.5	16	0.78 ± 0.36	4	0.26 ± 0.13	1	50
T242 (melanoma)	0.67 ± 0.25	13 ± 6.0	19	1.0 ± 0.30	4	0.26 ± 0.22	0.4	50
T24B (bladder)	0.39 ± 0.15	8.8 ± 4.8	22	0.52§	1	0.11§	0.3	ND
Hepatocarcinoma	0.21§	20§	95	ND		0.13§	0.6	ND
Osteosarcoma	0.23 ± 0.10	14§	61	ND		0.19 ± 0.20	0.8	50

* Cells (0.2×10^5/ml) were grown in Autopow media containing 10% fetal calf serum in the presence of drug in Linbro wells and were counted when control untreated cells reached a concentration of $1–2 \times 10^5$ cells/ml. Tumor lines were allowed to attach to plates 18–24 hr before addition of drugs. Each IC_{50} is an average of four to six experiments ± S.D. except where indicated.

† Equivalent to 5×10^{-6} M MTX-PLL 60K. ‡ Not done. § Average of two experiments.

fraction was separated from the lysate by centrifugation.

To eliminate the possibility that the use of trypsin to detach tumor cells from flasks could have affected the results reported here, the experiments were repeated using cells which had been mechanically detached from the flasks. Identical results were obtained.

RESULTS

In vitro *growth experiments with MTX, MTX–HSA and MTX–PLL 3K and 60K.* Table 1 shows the concentrations of MTX, MTX–HSA, and MTX–PLL 3K and 60K needed to inhibit growth of five lines of human lymphocytes and five lines of human tumor cells to 50 per cent of control untreated cells (IC_{50}), as well as the ratios of the IC_{50} of the MTX–carrier to the IC_{50} of free MTX for each line. The concentrations of free MTX ($2–7 \times 10^{-8}$) or MTX–HSA ($7–15 \times 10^{-7}$) needed to inhibit growth rate by 50 per cent in the two classes of cells did not differ significantly. The concentration of MTX–HSA required to inhibit cell growth was twenty to fifty times higher than that of free MTX. Both MTX–PLL 3K and 60K, however, showed significant differences in their toxicities to lymphocytes and tumor lines. With the lymphocytes, a 10- to 40-fold higher concentration of MTX–PLL 3K and a 6- to 17-fold higher concentration of MTX–PLL 60K was needed for 50 per cent growth inhibition relative to the IC_{50} of free MTX. With the tumor lines the IC_{50} for MTX–PLL 3K was only one to four times higher than IC_{50} of the free drug, and the IC_{50} for MTX–PLL 60K against the five tumor lines was equal or lower than free MTX. At concentrations of 25 μg PLL 60K/ml (equivalent to 5×10^{-6} M MTX–PLL 60K), the carrier itself becomes toxic, possibly due to leakage of small molecules from the cell [22]. PLL 3K was less toxic and became so only above 40 μg/ml (or in excess of equivalent concentrations of 10^{-5} M MTX–PLL 3K).

The data in Table 2 show that neither chloroquine (10^{-5} M) nor lupeptin (2.5×10^{-5} M), two inhibitors of the lysosomal protease cathepsin B [23, 24], significantly altered the IC_{50} of free MTX, MTX–HSA or the MTX–PLL derivatives for the WI-L2 lymphoblast line. They also had no effect on cell growth at the concentrations used. In contrast, for the malignant T24A astrocytoma line, although the presence of chloroquine or lupeptin did not significantly alter the IC_{50} for free MTX or MTX–HSA, they increased the IC_{50} for MTX–PLL 60K by almost 10-fold. The action of MTX–PLL 3K was considerably less affected by the presence of these lysosomal inhibitors, and methylamine, a compound which inhibits lysosomal proteolysis by altering the pH milieu in the lysosomes [25], was not able to reverse the toxicity of MTX–PLL 60K. These results show that whereas the cytotoxic effects of MTX and MTX–HSA on both tumor cells and lymphocytes were not affected by the presence of the lysosomal protease inhibitors, the same inhibitors significantly decreased the toxicity of MTX–PLL 60K toward tumor cells, but not toward the lymphocytes. This suggests that the lysosomal enzymes may play a role

Table 2. Effect of 10^{-5} M chloroquine or 2.5×10^{-5} M lupeptin on the IC_{50} values of MTX, MTX–HSA, and MTX–PLL 3K and 60K against WI-L2 and T24A cells*

Cells	MTX ($\times 10^{-7}$ M)	MTX–HSA ($\times 10^{-7}$ M)	MTX–PLL 3K ($\times 10^{-7}$ M)	MTX–PLL 60K ($\times 10^{-7}$ M)
WI-L2				
Control	0.28 ± 0.05	7.3 ± 1.0	6.8 ± 2.0	4.3 ± 1.2
+ Chloroquine	0.24 ± 0.07	7.0 ± 0.7	9.1 ± 1.8	6.8 ± 1.6
+ Lupeptin	0.24	6.9	8.9	6.5 ± 2.0
T24A				
Control	0.21 ± 0.02	2.5 ± 0.8	0.73 ± 0.9	0.26 ± 0.1
+ Chloroquine	0.23 ± 0.01	3.1 ± 1.1	1.3 ± 0.8	2.1 ± 0.2
+ Lupeptin	0.26 ± 0.01	2.4 ± 1.1	2.1 ± 1.0	2.2 ± 0.4
+ Methylamine (10^{-3} M)				0.28

* Cells were grown in Autopow media containing 10% fetal calf serum in Linbro wells in the presence of MTX or MTX derivative with or without inhibitor, starting at a concentration of 0.2×10^5 cells/ml. They were counted when control cells reached a concentration of $1–2 \times 10^{-5}$ cells/ml. Chloroquine, (10^{-5} M) lupeptin (2×10^{-5} M) or methylamine (10^{-3} M) did not inhibit cell growth at these concentrations.

in initiating breakdown of MTX–PLL 60K in the tumor cells and that this process is inhibited in the presence of the protease inhibitors.

The abilities of cells to recover their growth rate after a 2- or 24-hr exposure to MTX, MTX–HSA or the MTX–PLL derivatives are summarized in Table 3. At concentrations of MTX or MTX–HSA approximately ten to fifteen times their IC_{50} values, a 2-hr exposure did not impair the growth rate of either WI-L2 or T24A cells. The WI-L2 cells were also able to recover their growth rate after a 2-hr exposure to 5×10^{-6} M MTX–PLL 3K ($IC_{50} \times 10$). However, the growth of T24A cells was considerably impaired after a 2-hr exposure to MTX–PLL concentrations only two to six times their IC_{50} values. Thus, the effects of a short period of exposure to relatively low concentrations of MTX–PLL were not readily reversible in the tumor cells, whereas the same cells recovered their growth rate completely after a 2-hr exposure to much higher concentrations of MTX or MTX–HSA.

Inhibition of [^{3}H]*-dUrd incorporation into nucleic acids of WI-L2, T24A and human marrow cells by MTX, MTX–HSA and MTX–PLL.* Table 4 shows the concentrations of MTX and of the MTX–carrier complexes necessary to inhibit incorporation of [^{3}H]-dUrd into the nucleic acids of WI-L2, T24A and human marrow cells to 10–15 per cent of control incorporation after 4-hr of incubation with drugs. The degree of inhibition of [^{3}H]-dUrd incorporation into DNA reflects the extent of inactivation of the thymidylate synthesis cycle which occurs when the target enzyme dihydrofolate reductase is inhibited by MTX or its derivatives. The concentration of free MTX or MTX–HSA required to inhibit [^{3}H]-dUrd incorporation did not differ significantly for WI-L2,

Table 3. Effects of 2 and 24 hr of incubation with MTX, MTX–HSA and MTX–PLL 3K and 60K on growth of WI-L2 and T24A cells*

	Percentage of control growth rate							
	MTX		MTX–HSA		MTX–PLL 3K		MTX–PLL 60K	
	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
	5×10^{-7} M ($IC_{50} \times 15$)		10^{-5} M ($IC_{50} \times 15$)		5×10^{-6} M ($IC_{50} \times 10$)		6×10^{-7} M ($IC_{50} \times 2$)	
WI-L2	100	48	100	44	100	60	100	68
	5×10^{-7} M ($IC_{50} \times 15$)		10^{-5} M ($IC_{50} \times 15$)		5×10^{-7} M ($IC_{50} \times 5$)		5×10^{-8} M ($IC_{50} \times 2$)	
T24A	100	40	100	48	34	28	26	16

* Cells (0.2×10^5/ml) were incubated with MTX or MTX derivatives at 37° for 2 or 24 hr. The drug was then removed by centrifugation (WI-L2) or aspiration (T24A) and the cells were washed with PBS. They were then resuspended in fresh medium and grown until the control untreated cells had reached $1–2 \times 10^5$ cells/ml.

Table 4. Inhibition of [^{3}H]-dUrd incorporation by MTX, MTX–HSA, and MTX–PLL 3K and 60K in WI-L2, T24A and human bone marrow cells, and the ability of chloroquine to reverse these effects*

Cell line and treatment	Percentage of control [^{3}H]-dUrd incorporation			
	MTX	MTX–HSA	MTX–PLL 3K	MTX–PLL 60K
	(4×10^{-7} M)	(5×10^{-6} M)	(10^{-5} M)	(10^{-6} M)
WI-L2				
Without chloroquine	10 ± 6	17 ± 12	9 ± 4	70 ± 24
+ Chloroquine (10^{-4} M)	11 ± 3	26 ± 2	54 ± 10	65 ± 11
	(2×10^{-7} M)	(3×10^{-6} M)	(5×10^{-7} M)	(5×10^{-7} M)†
T24A				
Without chloroquine	10 ± 5	16 ± 7	6 ± 3	10 ± 6
+ Chloroquine (10^{-4} M)	10 ± 3	30 ± 5	92 ± 12	100
	(4×10^{-7} M)	(6×10^{-6} M)	(10^{-5} M)	(10^{-6} M)
Human bone marrow	10 ± 3	10 ± 4	44 ± 20	66 ± 8N

* Cells ($2–5 \times 10^5$/ml) were incubated with MTX or MTX derivatives at concentrations needed to inhibit [^{3}H]-dUrd incorporation to approximately 10 per cent of control incorporation in growth media (containing 10% fetal calf serum) at 37° for 4 hr. Drugs were then removed and the cells were suspended in fresh media without fetal calf serum and were incubated with 2 μCi [^{3}H]-dUrd for 15 min. The cells were then washed and the DNA was precipitated as described in Materials and Methods. Chloroquine at 10^{-4} M did not inhibit [^{3}H]-dUrd incorporation. Control untreated cells incorporated approximately 90,000 cpm/10^6 cells.

† Inhibition of [^{3}H]-dUrd incorporation by 5×10^{-7} M MTX–PLL 60K in T24A cells occurred only after incubation with drug for 4 hr.

T24A or bone marrow cells; chloroquine could partially reverse this inhibition in cells exposed to MTX–HSA, but not in those exposed to MTX.

Both of the MTX–PLL derivatives were able to inhibit [^{3}H]-dUrd incorporation into the nucleic acids of T24A cells at 5×10^{-7} M without affecting [^{3}H]-thymidine incorporation (unpublished results), and this inhibition could be completely reversed by the presence of chloroquine. Inhibition of [^{3}H]-dUrd incorporation in T24A cells by MTX–PLL 60K at 5×10^{-7} M was not observed after 2 hr with the drug and occurred only after 4 hr. If the drug-containing medium was removed after 2 hr and the cells were incubated with fresh medium for another 2 hr the same inhibition was observed, indicating that degradation of MTX–PLL 60K to an entity capable of inhibiting DHFR was an intracellular event and did not take place in the extracellular medium.

Considerable inhibition of [^{3}H]-dUrd incorporation by MTX–PLL 3K in the WI-L2 and marrow cells was seen only at much higher concentrations (10^{-5} M) and in the WI-L2 cell line chloroquine was only partially effective in reversing these effects. MTX–PLL 60K was unable to significantly inhibit

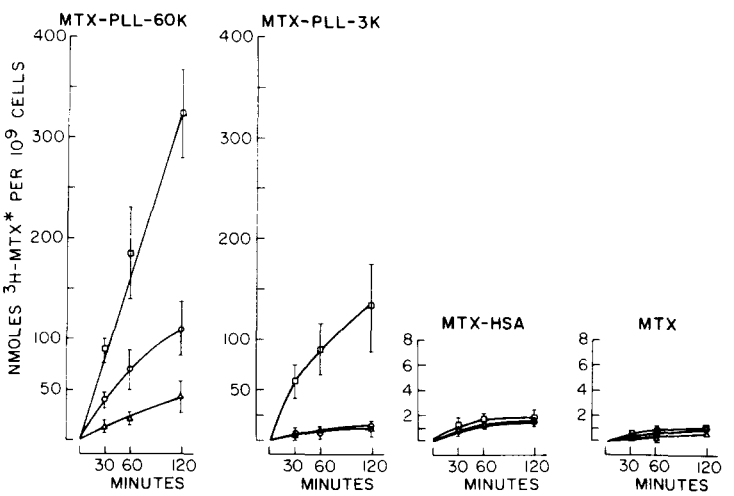

Fig. 1. Uptake of [^{3}H]-MTX-associated radioactivity (±S.D.) by T24A (□—□—□); WI-L2 (○—○—○); and human bone marrow cells (△—△—△) after incubation with 5×10^{-7} M [^{3}H]-MTX, [^{3}H]-MTX–PLL 3K and [^{3}H]-MTX–PLL 60K, and 5×10^{-6} M [^{3}H]-MTX–HSA. Cells (0.5 to 2×10^6/ml) were incubated with [^{3}H]-MTX or [^{3}H]-MTX derivatives at 37° in Autopow medium containing 10% fetal calf serum for 0.5, 1 and 2 hr. Cells were then washed to remove external radioactivity and counted as described in Materials and Methods. The asterisk refers to radioactivity equivalent to nmoles [^{3}H]-MTX/10^9 cells.

DHFR in WI-L2 and marrow cells at concentrations where the damaging effects of the carrier itself did not become apparent. At concentrations higher than 10^{-6} M this derivative began to interact with cells in a way that was unrelated to its antifolate activity. Thus, inhibition of DHFR by MTX–PLL 60K was exhibited only in the tumor cells and, in spite of substantial accumulation of the drug in WI-L2 cells during this time (Fig. 1), it remained in these cells in an inactive form.

Uptake of [^{3}H]*-MTX,* [^{3}H]*-MTX–HSA and* [^{3}H]*-MTX–PLL 3K and 60K by WI-L2, T24A and human bone marrow cells.* Figure 1 shows the uptake of [^{3}H]-MTX-associated radioactivity by WI-L2, T24A and human marrow cells after 0.5, 1 and 2 hr of incubation at 37° with 5×10^{-7} M [^{3}H]-MTX, [^{3}H]-MTX-PLL 3K or [^{3}H]-MTX–PLL 60K and 5×10^{-6} M [^{3}H]-MTX–HSA. After 2 hr of incubation with 5×10^{-7} M [^{3}H]-MTX, radioactivity equivalent to 0.5 to 1.1 nmoles MTX/10^9 cells was found in all cell lines. A 10-fold higher external [^{3}H]-MTX–HSA concentration (5×10^{-6} M) was needed to acheive comparable drug levels (radioactivity equivalent to 1.5 to 2.0 nmoles MTX/10^9 cells) with this derivative after 2 hr, indicative of its less efficient method of transport [26]. Again, uptake among the three cell lines did not differ significantly. The MTX–PLL derivatives were taken up in substantially greater amounts by all three cell lines when compared to the free drug (radioactivity equivalent to 320 nmoles [^{3}H]-MTX–PLL 60K and 120 nmoles [^{3}H]-MTX–PLL 3K/10^9 T24A cells, and 3 to 10-fold lower levels for WI-L2 and marrow cells). Figure 1 also shows that, whereas uptake of [^{3}H]-MTX and [^{3}H]-MTX–HSA by the T24A cells had reached saturability before 2 hr, uptake of the [^{3}H]-MTX–PLL derivatives by these cells at 2 hr was still continuing. These results indicate that conjugation of MTX to PLL increased the uptake of drug by all three cell lines over that of free MTX, confirming results reported previously for other cell lines [11]. They also show that, at physiological temperature, uptake of MTX–PLL 3K and 60K by the T24A tumor cells was significantly higher than uptake by WI-L2 or bone marrow cells, thus reflecting the IC_{50} of the MTX–PLL derivatives against these cells.

Figure 2 shows that at 0° the rate of uptake of the MTX–PLL derivatives by T24A cells was considerably reduced from its uptake rate at 37° but that, although this temperature-dependent uptake was also evident in uptake of MTX–PLL 60K by WI-L2 cells, there was no significant difference between the uptake of MTX–PLL 3K by WI-L2 cells at 0 and 37°. When WI-L2 or T24A cells were lysed after incubation with MTX–PLL at 0 or 37°, the majority of the [^{3}H]-MTX–PLL label (65–75 per cent) was found associated with the cell membrane fraction, suggesting that much of the drug was adsorbed to the outside of the cell membrane.

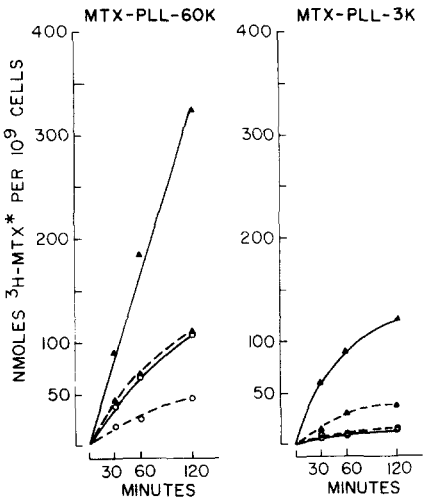

Fig. 2. Uptake of [^{3}H]-MTX-associated radioactivity by T24A cells at 37° (▲——▲) or 0° (▲----▲), and by WI-L2 cells at 37° (○——○) or 0° (○----○) after incubation with 5×10^{-7} M [^{3}H]-MTX–PLL 3K and [^{3}H]-MTX–PLL 60K. Cells (0.5 to 2×10^6/ml) were incubated with 5×10^{-7} M [^{3}H]-MTX–PLL 3K or [^{3}H]-MTX–PLL 60K at 37° or 0° in Autopow medium containing 10% fetal calf serum for 0.5, 1 and 2 hr. Cells were then washed to remove external radioactivity and counted as described in Materials and Methods. The asterisk refers to radioactivity equivalent to nmoles [^{3}H]-MTX/10^9 cells.

DISCUSSION

The results of this investigation indicate that, by coupling MTX to PLL, some measure of differential toxicity could be introduced between human lymphocytes on the one hand and human tumor cells on the other, under conditions where free MTX or another drug–carrier complex, MTX–HSA, showed no selectivity. Although growth inhibition studies could not be carried out with human bone marrow cells, the [^{3}H]-dUrd incorporation and uptake studies with these cells indicate that they resemble the WI-L2 cells rather than the T24A astrocytoma cell and, therefore, that some selective advantage might be anticipated *in vivo* between T24A and marrow cells if the drug were administered by continuous low dose infusion to maintain constant controlled serum concentrations.

The selective toxicity of MTX–PLL toward the T24A cells seems to have derived either from the increased uptake of this analog at physiological temperature relative to lymphocytes and marrow cells, or from the ability of these cells to convert MTX–PLL at relatively low concentrations either to free MTX or to an MTX derivative that was able to inhibit DHFR. It was shown previously that MTX–PLL 70K was ineffective in inhibiting DHFR [11].

The uptake studies with MTX–PLL show that all three cell lines were able to accumulate considerable amounts of the drug after a 2-hr incubation at 37°, with the greatest uptake shown by the T24A cells. At an external cell concentration of 5×10^{-7} M, the uptake of the MTX–PLL derivatives by T24A cells was 125–325 times the amount of drug found in cells incubated with the free drug and 15–100 times as much for the WI-L2 cells. Yet MTX–PLL was only slightly more toxic than MTX to T24A cells and considerably less toxic than the free drug toward the WI-L2 cells, indicating that much of the drug remains

either biochemically inactive in the cells or immobilized on the cell membrane. In contrast, the higher concentrations of MTX–HSA needed to inhibit cell growth can be correlated with low uptake of the MTX–HSA complex by both lymphocytes and tumor cells. The increased uptake of the MTX–PLL derivatives by T24A cells over WI-L2 or marrow cells may be due to the fact that PLL preferentially binds to cells that require surface attachment for growth, or to a more negatively charged membrane surface on T24A cells that would preferentially attract, in a non-specific way, a molecule such as PLL that is positively charged at physiological pH. Many types of malignant cells have been shown to have higher surface negative charge than normal cells [27–29]. If, on the other hand, the affinity of MTX–PLL for T24A cells is more specific, internalization at 37° may take place through receptor-mediated pinocytosis, a process involving coated pits or vesicles on the cell membrane [30] responsible for the selective uptake and removal of molecules such as α_2-macroglobulin and insulin [31].

In addition to the enhanced uptake of MTX–PLL by the T24A cells compared to the uptake by lymphocyte and marrow cells, the [^{3}H]-dUrd incorporation studies show that, whereas inhibition of DHFR occurred at 5×10^{-7} M MTX–PLL in the T24A cells, no inhibition of DHFR could be observed in WI-L2 or marrow cells with MTX–PLL 60K until concentrations were reached where the carrier itself became toxic. The ability of chloroquine and lupeptin, two inhibitors of the lysosomal protease cathepsin B [23, 24], to decrease the toxicity of MTX–PLL 60K toward the T24A cells and to reverse inhibition of [^{3}H]-dUrd incorporation into DNA without significantly affecting its uptake (unpublished results) indicates that lysosomal proteases may play some role in the breakdown of MTX–PLL in these cells. However, direct biochemical evidence that MTX–PLL is broken down by the T24A cell lysosomes or that this degradation can be reversed in these cells by chloroquine or lupeptin is lacking at present. In contrast to the MTX–PLL derivatives, the IC_{50} of MTX–HSA was not affected by chloroquine or lupeptin, and chloroquine was only minimally effective at protecting cells against inhibition of [^{3}H]-dUrd incorporation. Since the inhibition by MTX–HSA of purified DHFR was only 6- to 8-fold less than that of MTX [26], it may be less dependent than MTX–PLL on lysosomal degradation, or breakdown may occur preferentially in the cytoplasm. These experiments show that the mechanisms of action of these two carriers are different.

Past work has shown that PLL itself has anti-tumor activity toward the Ehrlich ascites tumor *in vivo* [32], and the results reported here show that at higher concentrations (23 μg/ml, or equivalent to more than 10^{-6} M MTX–PLL 60K) it is toxic to human cells *in vitro*, possibly due to leakage of ions from the cell and perturbation of cell surfaces [22]. This feature, as well as the facility with which PLL is taken up by normal cells, indicates that it is not an ideal carrier. In contrast, HSA, a major normal component of serum, is non-toxic as a carrier, but it is non-selectively and slowly taken up by the three cell lines used in these studies. The pharmacologic properties of the two drug–carrier complexes can therefore be expected to be very different *in vivo*. Whether the selective toxicity shown by the MTX–PLL derivatives toward the T24A tumor cells versus marrow cells *in vitro* can also be demonstrated under dynamic *in vivo* conditions and whether they are more therapeutically effective than free MTX or MTX–HSA against the same tumor cells transplanted *in vivo* into nude athymic mice are currently being investigated.

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