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SHORT COMMUNICATIONS

Treatment of Reuber H35 Hepatoma Cells with Carrier-Bound Methotrexate

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SUMMARY

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Methotrexate (MTX), 40 μM, covalently linked to bovine serum albumin (BSA), was ineffective in suppressing the growth of an MTX transport-resistant strain of Reuber hepatoma H35 cells ($I_{50(MTX)} \sim 3.5$ μM). However, conjugation of MTX with poly(L-lysine) led to cell growth repression at levels of <0.1 μM. In fact, both the parent H35 line and transport-resistant sublines showed a similar response to treatment with MTX[poly(L-lysine)] ($I_{50} \sim 70$ nM). Depressed cell growth after drug treatment of the H35 cells and the resistant sublines could be partially reversed by treatment with thymidine/hypoxanthine. Additionally, folinic acid was effective for preventing MTX[poly(L-lysine)] toxicity in H35 cells but could not do so for the MTX transport deficient sublines, presumably because of its inability to enter the cells. These data are consistent with the proposal that MTX[poly(L-lysine)] is toxic to both cell lines via a blockade of the one-carbon metabolic pathway.

Interest has recently developed in the possible therapeutic use of carrier-bound drugs to secure greater interaction between the drug and its target site of action. Examples of carriers include liposomes (1), polypeptides (2), polysaccharides (3), and antibodies (4), and of drugs daunomycin (5), MTX[3] (6), actinomycin D, and cytosine arabinoside (7). In particular, in this laboratory, MTX coupled to albumin has been shown to be equally as effective as free MTX in the treatment of the mouse L1210 leukemia (8) and superior to the free drug in controlling the Lewis lung carcinoma (9). More recently a closer examination of the interaction of the carrier-bound MTX and L1210 cells has suggested that the nature of the carrier is important for interaction with the cell surface and that a carrier-bound drug might be absorbed into the cell by a mechanism alternate to that of the free agent (10). Because of the therapeutic significance of these observations, it was decided to examine the interaction of MTX-BSA with a nonsystemic cellular source such as the MTX transport-resistant Reuber H35 hepatoma cells which had been developed in one of the authors' laboratories (11). However, MTX-BSA was ineffective in suppressing growth of these cells. Therefore, because of the two observations that carrier structure can determine cellular interaction, and that poly(L-lysine) possesses exceptional cell membrane-penetrating properties (12), MTX was bound to this carrier and the product was tested with the hepatoma cell line. The following preliminary report describes the comparative toxicity of the polymer-bound and free drug with this MTX transport-resistant cell line, and presents experimental data to show cell rescue with reagents which usually overcome blockade of the one-carbon metabolic pathway.

The following were obtained from commercial sources: Swims medium S77, horse serum, and fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.); MTX (Lederle Laboratories, Wayne, N. J.) was purified by DEAE-cellulose prior to use (11); crystalline BSA, poly(L-lysine) ($M_r \sim 35{,}000$), hypoxanthine, and thymidine (Sigma Chemical Company, St. Louis, Mo.); 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (Story Chemical Corporation, Muskegon, Mich.); folinic acid (ICN Pharmaceuticals, Covina, Calif.). Dihydrofolate was synthesized by the procedure of Blakely (13), and the concentrations of the folate derivatives and MTX were determined by their respective extinction coefficients (14). MTX-BSA was synthesized according to a

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[3] The abbreviations used are: MTX, methotrexate; BSA, bovine serum albumin.

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previously described procedure (8) in which MTX is coupled via a terminal carboxyl group to ε-amino groups contained in the albumin molecule.

MTX[poly(L-lysine)] was synthesized according to a procedure first outlined by Ryser and Shen (15), modified in the following way: 0.1 ml of a neutral aqueous solution of MTX (13 mg/ml) and 0.05 ml of an aqueous solution of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (200 mg/ml) were added to poly(L-lysine) (13.7 mg) dissolved in 0.5 ml of 0.01 M potassium phosphate, pH 7.2, containing 0.15 M sodium chloride. After stirring in the dark at room temperature for 2.5 hr, the solution was applied to a column of Sephadex G-25 (2.2 × 90 cm) which had been equilibrated with the reaction buffer solution. Elution was carried out with the same buffer and the eluent absorbance was monitored at 280 nm. Fractions comprising the initial peak absorbance were combined and dialyzed against water (6 liters) and then lyophilized. The yellowish flocculent residue (9.1 mg) contained 4.8 moles of MTX per mole of poly(L-lysine) when estimated by MTX absorbance at 370 nm [$\epsilon_{0.1\ N\ NaOH} = 7100$ (14)].

H-11-EC3 (designated H35 cells) derived from the Reuber H35 hepatoma (16, 17) were grown as previously described and the development of resistant sublines was outlined in the same publication (11). Methotrexate-resistant lines are indicated by the designation H35R with the subscript following the R indicating the concentration (micromolar) of MTX to which the cells are resistant. All of the cells employed in this study, unless otherwise noted, were resistant solely through defective transport and showed no more than 50% increase in dihydrofolate reductase relative to control cultures, whereas cells resistant to MTX at concentrations of 2 μM or greater demonstrated elevations in dihydrofolate reductase which correlated directly with resistance (11, 18).

The cells were cultured at a density of 5×10^4 cells/ml in 4 ml of Swims S77 medium with 20% horse serum and 5% fetal calf serum in a 95-5% air-CO_2 mixture at 37°. Drug additions were made to duplicate samples and the cultures were allowed to incubate for 72 hr after which the cells were released from the plates with 0.05% trypsin and counted with a ZB1 Coulter counter (11).

The comparative growth curves of H35 cells plated at 2×10^5 cells/dish in the presence of various levels of MTX-BSA[4] is illustrated in Fig. 1. Clearly the growth of wild-type cells was retarded by micromolar quantities of MTX-BSA with 40 μM MTX-BSA leading to a complete cessation of growth. However, the $H35R_1$ cells were unaffected by this concentration of carrier-bound drug and demonstrated a growth response comparable to that of the untreated H35 cells.

The influence of various concentrations of MTX[poly(L-lysine)] on both H35 and $H35R_{0.3}$ hepatoma cells is illustrated in Fig. 2. The effect is also compared with that of the free drug. MTX showed little restraint on the growth of the transport-resistant mutants at levels up to 1 μM in contrast to the wild-type cells which submit

[4] Unless stated otherwise, all weights reported for the carrier-bound derivatives of MTX refer to the quantity of ligand contained by the complex.

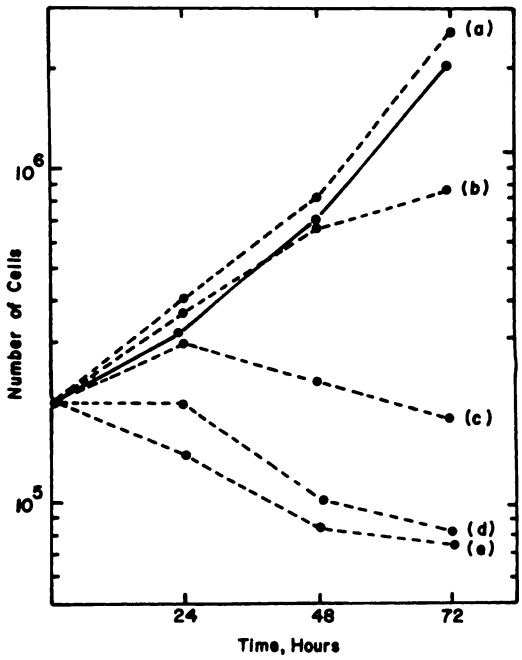

FIG. 1. *The comparative growth curves of H35 cells (●- - -●) plated at a density of 2 × 10^5 cells/dish in Swims medium S-77 supplemented with 20% horse serum, 5% fetal calf serum, and 4 mM glutamine*

The cells were treated with: (*a*), 0 μM; (*b*), 2 μM; (*c*), 5 μM; (*d*), 20 μM; and (*e*), 40 μM MTX-BSA. The response of MTX-resistant $H35R_{1.0}$ cells to treatment with 40 μM MTX-BSA when grown under conditions similar to those for the nonresistant cells is shown by ●——●.

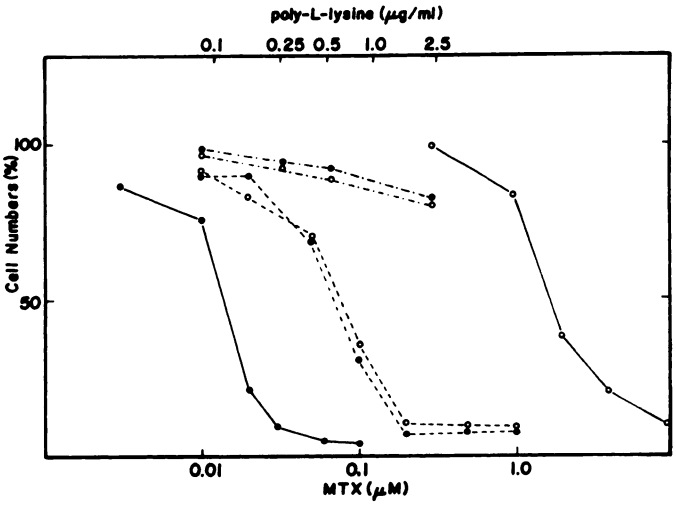

FIG. 2. *The comparative growth curves of resistant (○) $H35R_{0.3}$ and nonresistant (●) H35 cells when treated with MTX (——), MTX[poly(L-lysine)] (- - -), and poly(L-lysine) (-·-) alone at the levels indicated*

The growth rates are expressed as a percentage of those observed with untreated cell samples.

to nanomolar concentrations of the drug (I_{50} = 14 nM). However, when MTX was bound to the poly(L-lysine) carrier, both cell lines showed similar characteristics with $I_{50} \sim 70$ nM. The concentrations of poly(L-lysine) used in the experiments showed little influence on the cell growth. Interestingly, the level of substitution of the drug on the carrier appeared to influence the I_{50}. For example, a sample of MTX[poly(L-lysine)] containing 2.25 moles

of MTX per mole of carrier showed an I_{50} of 160 nM for resistant and normal cells, in contrast to the situation described in Fig. 2 where substitution was at a level of 4.8:1.

Table 1 shows the differential effects observed when H35 and H35R$_1$ cells treated with MTX (0.1 μM) or MTX[poly(L-lysine)] (1.0 μM) were rescued with folinic acid (100 μM) or thymidine/hypoxanthine (500/100 μM). The H35 cells were sensitive to both drugs and responded in each case to supplementation with folinic acid, although those treated with the free drug gave a better response than those treated with MTX[poly(L-lysine)] (92% as opposed to 69% of normal growth rate). The H35R$_1$ cells, unaffected by MTX but susceptible to MTX[poly(L-lysine)], did not respond to folinic acid. Both cell types also showed a moderate response to thymidine/hypoxanthine rescue. It is of note that less toxic levels of thymidine/hypoxanthine did not rescue the cells as efficiently.

The superior ability of MTX[poly(L-lysine)] to deliver MTX to the transport-resistant hepatoma mutants is illustrated in Table 2. That resistance is conferred by a deficiency in MTX transport can be discerned from the observation that only in the H35R$_6$ line did changing levels of dihydrofolate reductase, an alternate pathway for increased resistance of MTX toxicity, show an appreciable increment. In this experiment the I_{50} for the carrier-bound drug remained essentially constant from wild-type to 1 μM-resistant cells, whereas the free drug required a 100-fold increment in concentration to achieve the same mortality. An increment in MTX[poly(L-lysine)] requirement is only observed when the dihydrofolate reductase levels are increased.

When either MTX transport-defective or nondefective cells were treated with MTX and MTX[poly(L-lysine)], it was found that with both drugs, administered at the concentrations indicated in Fig. 3, the activity of dihydrofolate reductase in cell extracts declined to levels which could not be detected by the assay procedure (19) and that the levels of *de novo* thymidine synthesis measured by ^{3}H release from [5-^{3}H]deoxyuridine correspondingly decreased to very low levels.

TABLE 1

Prevention of MTX[poly(L-lysine)] toxicity to H35 and H35R$_1$ cells by folinic acid and thymidine-hypoxanthine

MTX[poly-(L-lysine)] (1.0 μM)	MTX (0.1 μM)	Folinic acid (100 μM)	Thymidine/ hypoxanthine (500 μM–100 μM)	Cells[a] (%)
		H35		
+				9
+		+		69 (64)
		+		107
+			+	36 (73)
			+	49
	+	+		92 (86)
	+			6
		H35R$_1$		
+				15
+		+		20 (21)
		+		98
+			+	40 (71)
			+	56

[a] Numbers of cells are expressed relative to the untreated cultures. The numbers in parentheses refer to the extent of cell growth observed in the presence of the inhibitor and protective agent relative to the growth of the cells in the presence of the protective agent alone.

TABLE 2

The concentrations of MTX and MTX[poly(L-lysine)] required to inhibit the cell division of H35, H35R$_1$, and H35R$_6$ cells.

The levels of dihydrofolate reductase present in each cell type were also measured.

Cell line	I_{50}[a]		Dihydrofolate reductase[b]
	MTX	MTX[poly-(L-lysine)][c]	
	nM		nmoles min^{-1} mg^{-1}
H35	15	105	7.5
H35R$_1$	2,000	130	9.0
H35R$_6$	10,000	600	43.0

[a] Indicates the concentration needed to cause a 50% inhibition of cell growth after 72 hr in culture.

[b] Assayed by a modification (19) of the method of Mathews (20).

[c] Ratio of MTX/poly(L-lysine) was 2.84/1.

The results presented in this report amplify the observation that carrier structure is integral to the concept of polymer-mediated drug delivery. Two additional features appear to be emerging: that a carrier-bound drug in a whole animal experiment can ensure that elevated levels of the drug are held compartmentalized within a body cavity and thus provide a continuously higher level of drug delivery (8, 9) and that polymer-bound drugs can overcome transport blocks which certain cells use to prevent passage of the free drug into the cell. The potential application of MTX[poly(L-lysine)] was suggested by its toxicity against chinese hamster ovary cells (15). We have applied this derivative to a cell line derived from a chemically induced hepatoma and its transport-resistant counterpart. The results demonstrate that the carrier MTX bypasses the transport defect, but is slightly less effective than MTX against the parent cell line. It has also been shown that the cells subject to chemotherapy with the MTX[poly(L-lysine)] can be rescued with hypoxanthine/thymidine but not with folinic acid; this observation suggests that, in addition to the action of MTX[poly(L-lysine)] via an antifolate mechanism, hepatomas may also be susceptible to the current destroy-rescue principle common in chemotherapy, if the optimal level of carrier-drug complex can be determined. The observation that folinic acid does not reverse toxicity in the resistant cells is consistent with the inability of transport mutants to take up either MTX or the reduced folates (18), as has already been shown with MTX or pyrimethamine toxicity in MTX transport-defective L5178Y lymphoblasts (22). The suggestion that the carrier-bound drugs act by an antifolate mechanism is further enhanced by the obvious decline in dihydrofolate reductase activity and ^{3}H release from deoxyuridine (Fig. 3) when extracts of cells are treated with MTX[poly(L-lysine)].

We intend to extend these investigations to hepatomas in whole animal experiments to determine whether an altered pattern of response to drug therapy is observed

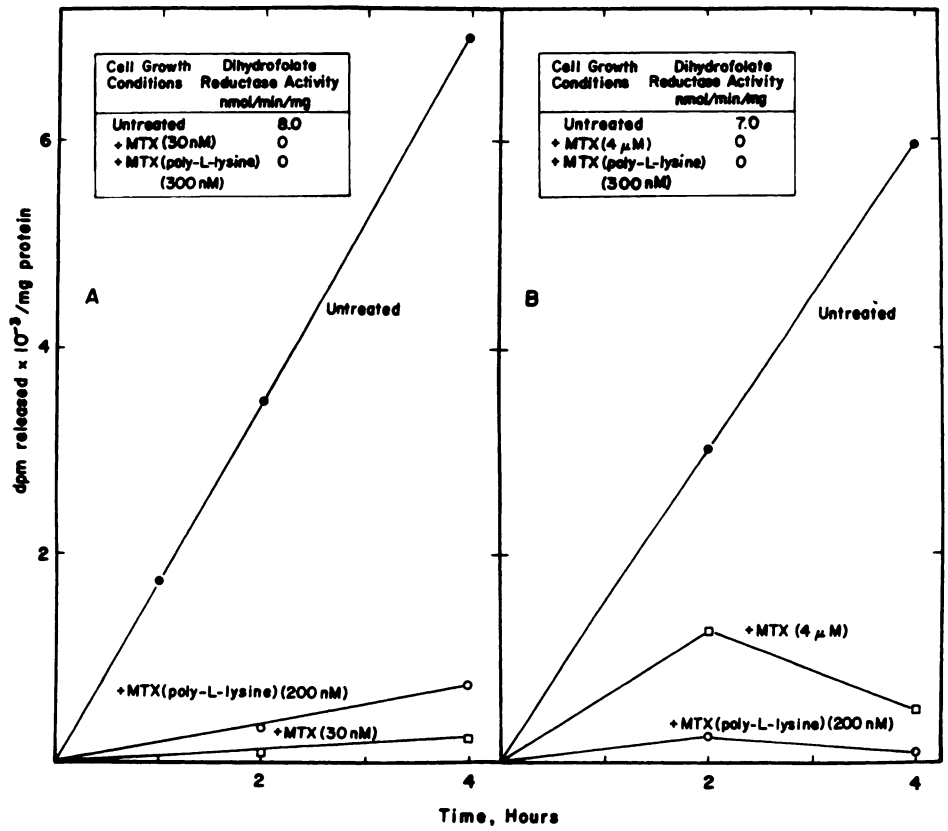

FIG. 3. *Inhibition of dihydrofolate reductase and de novo thymidylate biosynthesis by H35 cells after treatment with MTX and MTX[poly(L-lysine)]*

H35 (A) and $H35R_{0.3}$ (B) cells were plated as described in the text in the presence or absence of the indicated concentrations of MTX or MTX[poly(L-lysine)]. After 72 hr, dihydrofolate reductase activity was measured as described in Table 2. Extracts of the untreated cells were prepared from two 60-mm plates, and four 100-mm plates were used for the extracts of inhibited cells. Relative levels of *de novo* thymidylate synthesis were measured by the release of ^3H from [5-^3H]deoxyuridine as described in an earlier publication (21).

with the free and bound drugs. It is apparent, however, that poly(L-lysine) rather than albumin should be the carrier of choice for drugs used in the treatment of hepatoma cells showing any sign of a transport blockade—an observation which may also apply to other transformed cell types.

REFERENCES

1. Gregoriadis, G. The carrier potential of liposomes in biology and medicine. *N. Engl. J. Med.* **295**:704–710, 765–770 (1976).
2. Barbanti-Brodano, G., and L. Fiume. The selective killing of macrophages by amanitin-albumin conjugates. *Nature (Lond.)* **243**:281–283 (1973).
3. Harding, N. G. L. Amethopterin linked covalently to water-soluble macromolecules. *Ann. N. Y. Acad. Sci.* **186**:270–283 (1971).
4. Rowland, G. F. Effective antihuman conjugates of alkylating drug and antibody using dextran as the intermediate carrier. *Eur. J. Cancer* **13**:593–596 (1977).
5. Hurwitz, E., R. Maron, A. Bernstein, M. Wilcheck, M. Sela, and R. Arnon. The effect *in vivo* of chemotherapeutic drug-antibody conjugates in two murine experimental tumor systems. *Int. J. Cancer* **21**:747–755 (1978).
6. Fung, W.-P., M. Przybylski, H. Ringsdorf, and D. S. Zaharko. *In vitro* inhibitory effects of polymer-linked methotrexate derivatives on tetrahydrofolate dehydrogenase and murine L5178Y cells. *J. Natl. Cancer Inst.* **62**: 1261–1264 (1979).
7. Szekerke, M., and J. S. Driscoll. The use of macromolecules as carriers of antitumor drugs. *Eur. J. Cancer* **13**:529–537 (1977).
8. Chu, B. C. F., and J. M. Whiteley. High molecular weight derivatives of methotrexate as chemotherapeutic agents. *Mol. Pharmacol.* **13**:80–88 (1977).
9. Chu, B. C. F., and J. M. Whiteley. Control of solid tumor metastases with a high-molecular-weight derivative of methotrexate. *J. Natl. Cancer Inst.* **62**: 79–82 (1979).
10. Chu, B. C. F., and J. M. Whiteley. The interaction of carrier-bound methotrexate with L1210 cells. *Mol. Pharmacol.* **17**:382–387 (1980).
11. Galivan, J. H. Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. *Cancer Res.* **39**:735–743 (1979).
12. Shen, W.-C., and H. J.-P. Ryser. Conjugation of poly-L-lysine to albumin and horseradish peroxidase: a novel method of enhancing the cellular uptake of proteins. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1872–1876 (1978).
13. Blakely, R. L. Crystalline dihydropteroylglutamic acid. *Nature (Lond.)* **188**: 231–232 (1960).
14. Blakely, R. L. *The Biochemistry of Folic Acid and Related Pteridines* (A. Neuberger and E. L. Tatum, eds.). North-Holland Publishing Company, Amsterdam, 92–94 (1969).
15. Ryser, H. J.-P., and W.-C. Shen. Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3867–3870 (1978).
16. Pitot, H., C. Periano, P. Moose, and V. R. Potter. Hepatomas in tissue culture compared with adapting liver *in vivo*. *Natl. Cancer Inst. Monogr.* **13**:229–245 (1964).
17. Reuber, M. D. A transplantable bile secreting hepatocellular carcinoma in the rat. *J. Natl. Cancer Inst.* **26**:891–899 (1964).
18. Galivan, J. 5-Methyltetrahydrofolate transport by hepatoma cells and methotrexate-resistant sublines in culture. *Cancer Res.* **41**:1757–1762 (1981).
19. Bonney, R. J., and F. Maley. Effect of methotrexate on thymidylate synthetase in cultured parenchymal cells isolated from regenerating rat liver. *Cancer Res.* **35**:1950–1956 (1975).
20. Mathews, C. K. Evidence that bacteriophage-induced dihydrofolate reductase is a viral gene product. *J. Biol. Chem.* **242**:4083–4086 (1967).
21. Galivan, J. Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. *Mol. Pharmacol.* **17**:105–110 (1980).
22. Goldie, J. H., S. I. Harrison, L. A. Price, and B. T. Hill. Impaired responsiveness to folinic acid protection in methotrexate-resistant L5178Y cells. *Eur. J. Cancer* **11**:627–632 (1975).

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