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Poly (L-Lysine) and Poly (D-Lysine) Conjugates of Methotrexate: Different Inhibitory Effect on Drug Resistant Cells

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SUMMARY

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Conjugation of methotrexate to poly(L-lysine) markedly increases its cellular uptake and offers a new way to overcome drug resistance related to deficient transport. Conjugates using poly(L-lysines) of molecular weights varying from 3,100 to 130,000 have comparable effects on a drug resistant CHO cell line. Conjugates using poly(D-lysine), however, have no effect on either resistant or normal CHO cells. Both the L- and D-isomeric conjugates are taken up by cells in comparable fashion. Since even the biologically active conjugates are poor inhibitors of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase; EC 1.5.1.3) *in vitro*, it is concluded that the conjugate must give rise in the cell to a pharmacologically active breakdown product. Such a product can be detected in cells exposed to the L-isomeric, but not in cells exposed to the D-isomeric conjugates. Excess of poly(L-lysine) only moderately decreases methotrexate-poly(L-lysine) uptake but markedly decreases its inhibitory effect on cell growth.

INTRODUCTION

Poly(L-lysine) is avidly taken up by cultured cells and can be used as carrier for small as well as large molecules. When a 6,700 MW fragment of poly(L-lys)[2] is conjugated to horseradish peroxidase, it enhances by more than 400-fold the cellular uptake of active enzyme (1, 2). When used as carrier for covalently bound methotrexate, a 70,000 MW poly(L-lys) increases drug transport both in cells that are proficient and deficient in MTX transport. In the latter instance, a 200-fold increase in drug uptake overcomes the drug resistance due to transport deficiency (3). We have conjugated MTX to 60,000 MW poly(D-lysine) and to poly(L-lys) of 4 different sizes and tested these conjugates on 3 lines of Chinese hamster ovary cells. In this paper we present evidence that the growth inhibitory effect of MTX-poly(L-lys) requires the intracellular breakdown of the conjugate with subsequent liberation in the cell of a small pharmacologically active MTX adduct. We conclude that the biological effect

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[2] The abbreviations used are: poly(L-lys), poly(L-lysine); poly(D-lys), poly(D-lysine); MTX, methotrexate; DHFR, dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxydoreductase; EC 1.5.1.3); MTX-poly(L-lys), methotrexate conjugated to poly(L-lysine); MTX-poly(D-lys), methotrexate conjugated to poly(D-lysine); ^{3}H-MTX, [3', 5', 9-^{3}H] methotrexate; BSS, balanced salt solution; CHO, Chinese hamster ovary cell line.

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of MTX-poly(L-lys) and MTX-poly(D-lys) is limited not only by cellular uptake but also by the intracellular digestion of the conjugate. A preliminary report of this work has been presented (4).

MATERIALS AND METHODS

MTX was obtained from Sigma Chemical C., St. Louis, MO and [^{3}H] methotrexate sodium salt (250 μCi, 18.5 Ci/mmole) was purchased from Amersham Co., Arlington Heights, IL. Poly(L-lysine) hydrobromide, MW 130,000, 70,000, and 20,000, as well as poly(D-lysine) hydrobromide, MW 60,000 were purchased from Pilot Chemicals, Watertown, MA. Poly(L-lysine) hydrobromide, MW 3,100 was purchased from the New England Nuclear Corporation, Boston, MA. Heparin, dihydrofolate reductase from bovine liver and the chemicals required for its assay were purchased from the Sigma Chemical Co., St. Louis, MO. DHFR activity was measured by the method of Stanley *et al.* (5). The tissue culture products were purchased from Grand Island Biological Co., Grand Island, NY. Two Chinese hamster ovary cell lines, CHO Pro$^-$3 Mtx^{RII} 5-3 and CHO Pro$^-$4 MTX^{RII} 4-5, characterized as MTX-resistant due to transport deficiency (6, 7), were obtained from W. F. Flintoff, University of Western Ontario, London, Ont. They were grown as previously described (3).

Preparation of MTX-poly(L-lys) and MTX-poly(D-lys). MTX conjugates of poly(L-lys) hydrobromide, MW 70,000 and of poly(D-lys) hydrobromide (MW 60,000) were prepared as previously described (3) by the carbodiimide catalyzed coupling of a carboxyl group of MTX with an amino group of polylysine. A MTX-poly(L-lys) conjugate of MW 70,000 was hydrolyzed in 6 N HCl and subjected to amino acid analysis. The ratio of glutamic acid to lysine in this conjugate was found to be 1:27, which indicates that one in 27 lysyl residues was covalently linked to MTX. Since a poly(L-lys) hydrobromide of 70,000 MW contains about 350 lysines, this ratio is in good agreement with our previous estimate, that the conjugate had approximately 13 molecules of methotrexate per molecule carrier (3). All the data presented here, except those of Table 1, were obtained with MTX-poly(L-lys) and MTX-poly(D-lys) of 70,000 and 60,000 molecular weight, respectively.

MTX conjugates of poly(L-lys) of 3,100, 20,000 and 130,000 MW were prepared by the same method. These conjugates were purified by either Sephadex G-50 or Sephadex G-25 gel filtration. The amounts of MTX per mg of poly(L-lys) were almost identical in all four conjugates. However, in the case of conjugates with 3,100 molecular weight, aggregates were found in the concentrated solution. Therefore, the stock solution of this conjugate was filtered and the concentration of MTX was measured before the addition to the cell culture medium.

Determination of cellular degradation products in cultured cells. Methotrexate resistant cells, Pro$^-$3 Mtx^{RII} 5-3, were grown in a flask (75 cm^2) and were treated with 1×10^{-6} M of ^{3}H-MTX-poly(L-lys) one day before reaching confluence. After a 24-hour exposure, the growth medium containing the radioactively-labeled conjugate was removed and the cell monolayer was washed twice with 15 ml balanced salt solution. Cells were detached by brief trypsinization, and collected by low speed centrifugations. The cell pellet was washed three times with 5 ml BSS and was dissolved in 1 ml of 0.01 M sodium phosphate buffer, pH 7 with 1% sodium dodecyl sulfate. A small amount of unlabeled MTX (0.2–0.3 mg) was added before cell lysis as the carrier for ^{3}H-MTX in the cell extract. The cell solution was then loaded onto a Sephadex G-25 column (1.5 × 24 cm) which had been equilibrated with phosphate-SDS buffer and the column was eluted with the same buffer. Each 2 ml fraction was collected from the eluent of the column and 0.5 ml aliquot from each fraction was counted in a liquid scintillation counter with 5 ml Aquasol. The unlabeled MTX that was added to the cell extract as carrier was detected by the measurement of absorbance at 257 nm.

Cellular uptake of ^{3}H-MTX and its poly(L-lys) or poly(D-lys) conjugates by CHO Pro$^-$3 Mtx^{RII} 5-3 cells. Monolayers of CHO Pro$^-$3 Mtx^{RII} 5-3 cells were exposed for 60 minutes at 37° to 1×10^{-6} M ^{3}H-MTX given as free or conjugated drug as previously described (3). The L- and D-

isomeric conjugates had carrier moieties of 70,000 and 60,000 MW, respectively. To eliminate the surface-bound conjugates, the detached cells were first washed for 5 minutes at 37° in 5 ml of BSS containing 5 mg/ml heparin (8). They were subsequently washed 3 more times in BSS at room temperature. The pellet of washed cells was then dissolved in 1 N NaOH. The experiments measuring the uptake of ^{3}H-MTX-poly(L-lys) in presence of a 10-fold excess of poly(L-lys) were carried out with larger culture flasks (75 cm^2) and used a lower MTX concentration (1×10^{-7} M). At that concentration, the poly(L-lys) content is 0.5 μg/ml; the incubations were therefore carried out in presence or absence of 5 μg/ml unlabeled poly(L-lys) of the same molecular weight.

Measurement of growth inhibitory effect of MTX and MTX-poly(L-lys) on MTX-transport proficient and deficient cells. The growth inhibitory studies were done on three CHO cell lines, namely the wild type, WTT, and the 2 mutants, Pro^-3 Mtx^{RII} 5-3 and Pro^-4 Mtx^{RII} 4-5. The three cell lines were seeded with 5×10^4 cells per tissue culture flask (25 cm^2) and were first grown for 24 hours in normal growth medium. The monolayers were then exposed for 4 days to culture medium containing various concentrations of MTX given either as free or conjugated drug, as previously described (3). The experiments measuring the growth inhibitory effect of MTX-poly(L-lys) in the presence of an excess of unconjugated poly(L-lys) or poly(D-lys) were carried out at only one MTX concentration, i.e., 1×10^{-7} M. That concentration corresponds to a content of 0.5 μg/ml poly(L-lys) of 70,000 MW. Unconjugated poly(L-lys) of 70,000 MW and poly(D-lys) of 60,000 MW were added in amounts of 0.5 to 4.0 μg/ml. The cells were counted after the 4 day exposure to the drug and their number was expressed as percent of the control cells grown in the absence of conjugate.

RESULTS

We showed previously that MTX-poly(L-lys) can inhibit the growth of CHO cells at MTX concentrations which are ineffective when given as free drug (3). This result, obtained with Pro^-3 Mtx^{RII} 5-3, has now been confirmed using a second transport deficient mutant, Pro^-4 Mtx^{RII} 4-5, described by Flintoff and Saya (6, 7). These two cell lines are compared with their parental wild type in Fig. 1, with regard to their response to increasing concentrations of MTX and MTX-poly(L-lys). It is apparent that 40 and 70 times higher concentrations of free MTX are required for comparable growth inhibition of the two transport-deficient lines. Conjugation to poly(L-lys), however, reduces the difference in the dose causing a 50% inhibition (ID_{50}) to a factor of 2 and 3, respectively. Under the conditions of our experiments, the inhibitory effect of MTX conjugate on the wild type line is slightly less than that of the free drug and the ID_{50}'s differ by a factor of 3. While most of our data were obtained with a conjugate containing poly(L-lys) of MW 70,000, it is clear that carriers of other sizes can be as effective. As shown in Table 1, a poly(L-lys) of MW as small as 3,100 appears to retain adequate drug carrier properties after conjugation and may have some advantages for *in vivo* studies.

The marked inhibitory effect of MTX-poly(L-lys) on cell growth is in sharp contrast with its minimal effect on DHFR activity *in vitro*. As shown in Fig. 2, MTX is a very potent inhibitor of DHFR in its free form while its poly(L-lys) conjugate is 3 orders of magnitude less effective. The concentrations which cause 50% inhibition of enzyme activity are 5×10^{-9} M and 2×10^{-6} M for the free and conjugated drug, respectively. It is conceivable that the remaining activity of the conjugate may be due to traces of free MTX, which would suggest that the conjugate was 99.75% pure. The addition of free poly(L-lys) up to 50 μg/ml did not interfere with the enzyme assay. To test whether this loss of activity might be restored by hydrolysis of the conjugate, MTX-poly(L-lys) was incubated overnight at 37° in the presence of .025% trypsin. As indicated in Fig. 2, the tryptic digest had intermediate inhibitory activity. Similar recoveries were obtained when the conjugate was exposed for 1 hour at 37° to pronase. Chromatography of the pronase digest of

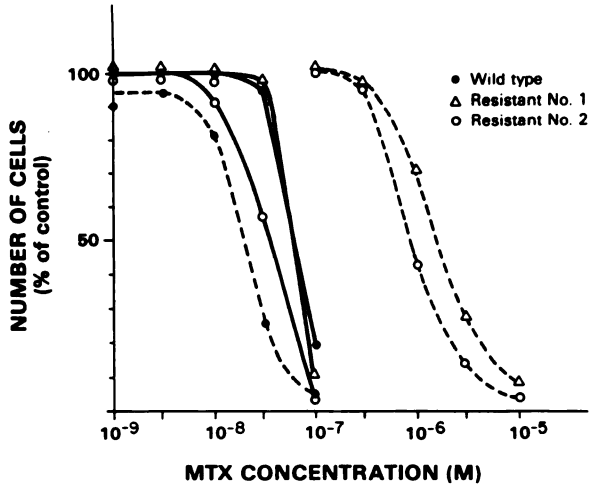

FIG. 1. *Growth inhibitory effects of MTX and MTX-poly(L-lys) MW 70,000 on three cultured CHO cell lines*
The drug sensitive, wild type CHO WTT (●), and two transport deficient and drug resistant mutants, Pro⁻3 Mtx^{RII} 5-3 (○) and Pro⁻4 Mtx^{RII} 4-5 (△), were treated with increasing concentrations of either MTX (- - - -) or MTX-poly(L-lys) (——). Cell number in each flask was counted after a 4 day exposure to MTX and was compared to the untreated control. The number of cells in the control flasks were 8.7×10^6, 6.9×10^6 and 5.6×10^6 for the WTT, Pro⁻3 and Pro⁻4 lines, respectively.

TABLE 1

Growth inhibitory effects of MTX conjugated to poly (L-lys) of different molecular weights

The MTX content per mg poly (L-lys) was comparable in all cases. The number of Pro⁻3 Mtx^{RII} 5-3 cells was counted at the end of a 4-day exposure to 1×10^{-7} M MTX-poly(L-lys). The control flask contained 6.2×10^6 cells.

Poly(L-lys) MW	Number of cells (%)
Control	100
130,000	9.7
70,000	11.7
20,000	7.1
3,100	7.0

^{3}H-MTX-poly(L-lys) revealed a radioactive peak of small molecular size eluting slightly before free MTX.

A comparison of the data of Figs. 1 and 2 suggests that MTX-poly(L-lys) must be activated once ingested by CHO cells. The hypothesis that the conjugate is digested inside the cells and gives rise to pharmacologically active breakdown products was tested in the following manner. Transport-deficient CHO cells were exposed for 24 hours to 1×10^{-6} M ^{3}H-MTX-poly(L-lys) and thoroughly washed with BSS and heparin-containing BSS before being lysed in

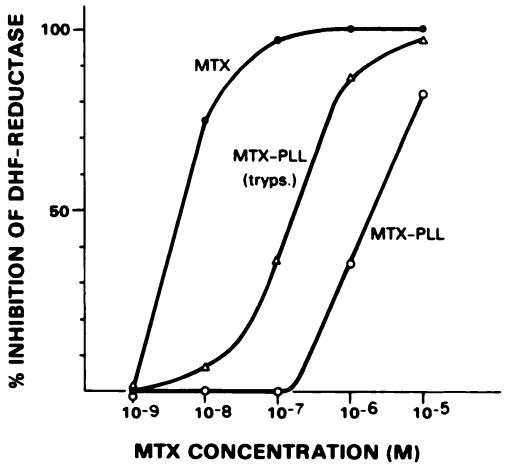

FIG. 2. *The inhibitory effect of MTX and MTX-poly(L-lys) MW 70,000 on dihydrofolate reductase*
DHFR activity was measured by the method of Stanley, *et al.* (5) in the presence of MTX (●), MTX-poly(L-lys) (○) or MTX-poly (L-lys) incubated overnight with 0.025% trypsin (△).

1% sodium dodecyl sulfate. The cell extract was then chromatographed on a Sephadex G-25 column and the eluate was monitored for both its radioactivity and MTX-content. The elution profile is shown in Fig. 3. The arrows correspond to the total volume of the column. The solid curves show the

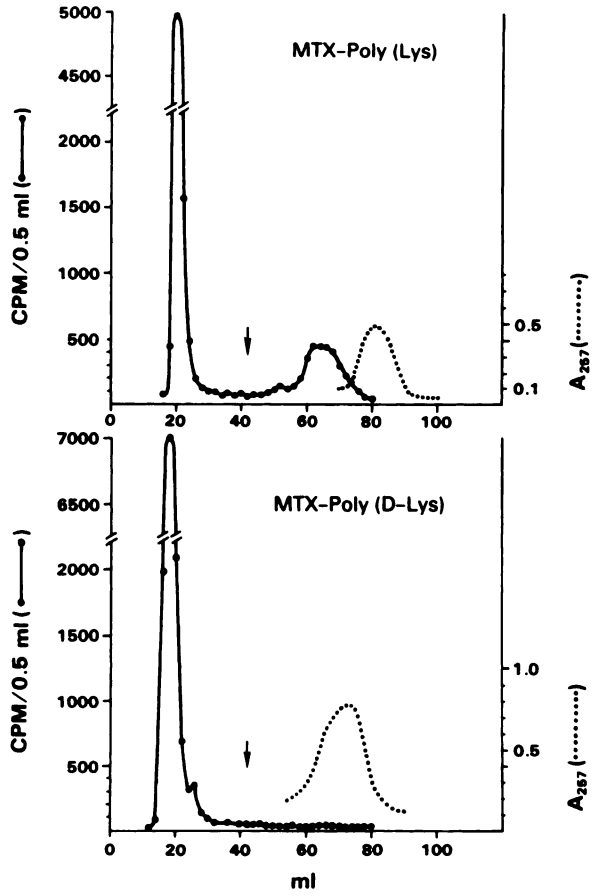

FIG. 3. *Intracellular degradation of MTX-poly(L-lys) MW 70,000 and MTX-poly(D-lys) MW 60,000 by CHO Pro⁻3 Mtx^{RII} 5-3 cells exposed for 24 hr to 1 × 10⁻⁶ M of ^{3}H-MTX-poly(L-lys) (upper panel) or ^{3}H-MTX-poly(D-lys) (lower panel)*

Cells lysates were chromatographed on a Sephadex G-25 column as described in MATERIALS AND METHODS. Arrows indicate the column volume. The elution of unlabeled carrier-MTX was determined by absorption at 257 nm (- - - -). A radioactive degradation product is found to elute close to MTX (—●—).

radioactivity in each fraction, and the dotted curves indicate the unlabeled MTX added to the cell extract as carrier and internal marker. The extract from MTX-poly(L-lys)-treated cells shows a peak of radioactivity representing about 25% of the total, which elutes close to but somewhat before the peak of MTX. This small molecular material has not yet been identified, but is believed to be a digestive product of the conjugate, presumably a MTX-lysine or MTX-oligolysine, responsible for the growth inhibitory effect. The radioactivity eluted at void volume suggests that 75% of the cell-associated ^{3}H-MTX-poly(L-lys) was intact after 24 hours exposure. It is not likely that this initial peak contains enzyme-bound MTX, since chromatography was carried out in the presence of 1% SDS. No intermediate MTX-peptides could be detected.

Unlike its L-isomer, poly(D-lys) is not susceptible to common proteolytic enzymes. It was of interest to test whether it could substitute for poly(L-lys) as a carrier for MTX. MTX-poly(D-lys) of 60,000 MW containing the same amount of MTX per unit weight was compared with MTX-poly(L-lys) and free MTX for its effect on the growth of Pro⁻3 Mtx^{RII} 5-3 and CHO WTT cells. Figure 4 gives the results of a 4 day exposure to a uniform MTX concentra-

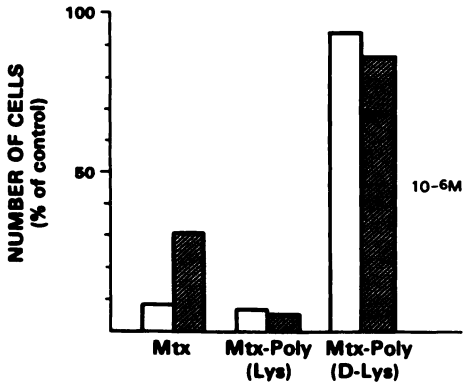

FIG. 4. *Growth inhibitory effects of MTX, MTX-poly(L-lys) MW 70,000 and MTX-poly(D-lys) MW 60,000 on cultured CHO cell lines*

Cells were grown in presence of 1×10^{-6} M MTX given either as free drug or as conjugated drug. Hatched column: MTX-resistant cells [Pro$^-$3 Mtx^{RII} 5–3]. Open column: Wild type, MTX-sensitive cells WTT. The control flasks contained 4.8×10^6 and 4.9×10^6 cells for the WTT and Pro$^-$3 lines, respectively.

tion of 1×10^{-6} M. At that concentration, in agreement with the data of Fig. 1, free MTX caused a 70% inhibition of transport deficient cells, while MTX-poly(L-lys) caused a near total growth inhibition of both transport proficient and deficient cells. The poly(D-lys) conjugate, however, had no inhibitory effect on either cell line. When Pro$^-$3 Mtx^{RII} 5–3 cells were exposed for 24 hours to ^{3}H-MTX-poly(D-lys) and examined for their content of small molecular ^{3}H-MTX derivatives, no such component was detected in their cell extracts (Fig. 3 lower panel).

The difference in the pharmacologic effect of the poly(L-lys) and poly(D-lys) conjugates is not due to differences in their cellular uptake. When transport deficient cells were exposed for 1 hour to comparable concentrations of ^{3}H-MTX-poly(L-lys) and ^{3}H-MTX-poly(D-lys), they took up similar amounts of the two conjugates. Table 2 compares the cell bound activity measured after 1 and 61 minute exposures to ^{3}H-MTX or ^{3}H-MTX conjugates. Net uptake of the poly(L-lys) conjugates, expressed by the difference between 61 and 1 min, was 200 times greater than that of free drug, confirming prior data (3). The net uptake of the L-lys and D-lys conjugates, however,

TABLE 2

Cellular uptake of ^{3}H-MTX, ^{3}H-MTX-poly(L-lys) and ^{3}H-MTX-poly(D-lys)

Pro$^-$3 MTX^{RII} 5–3 cells were exposed to ^{3}H-MTX or ^{3}H-MTX conjugates. The difference in cell-associated radioactivity measured after a 61 and 1 min exposure is used as an expression of net drug uptake in 1 hr. The poly(L-lys) and poly(D-lys) used as carriers had molecular weights of 70,000 and 60,000, respectively.

	cpm/mg cell protein
[MTX] 1×10^{-6} M	
^{3}H-MTX	33
^{3}H-MTX-poly(L-lys)	6089
^{3}H-MTX-poly(D-lys)	6409
[MTX] 1×10^{-7} M	
^{3}H-MTX-poly(L-lys)	643
^{3}H-MTX-poly(L-lys) + 5 μg/ml poly (L-lys)	519

was of comparable magnitude. The non-significant elevation in cell-bound ^{3}H-MTX-poly(D-lys) activity appears to be related to the use of trypsin to detach the monolayers; because the trypsin will break down poly(L-lys) but not poly(D-lys), it can be expected to decrease the amount of surface-bound poly(L-lys) in monolayers exposed to ^{3}H-MTX-poly(L-lys) for either 1 or 61 min.

Table 2 also shows the effect of a 10-fold excess of unlabeled poly(L-lys) on the uptake of ^{3}H-MTX-poly(L-lys). At a MTX concentration of 1×10^{-7} M, the conjugate contains 0.5 μg/ml poly(L-lys) of 70,000 MW. The addition of 5.0 μg/ml poly(L-lys) decreased by 20% the cellular uptake of ^{3}H-MTX-poly(L-lys). This suggests that poly(L-lys) can compete with ^{3}H-MTX-poly(L-lys) for uptake but only moderately so.

By contrast, even moderate excesses of poly(L-lys) profoundly influenced the pharmacologic effect of MTX-poly(L-lys) during a 4-day growth period. As was shown by Fig. 1, the growth of Pro$^-$3 Mtx^{RII} 5–3 cells is markedly (95%) inhibited by a 4-day exposure to 1×10^{-7} M MTX-poly(L-lys). This inhibition, however, is counteracted by the addition of free poly(L-lys) to the growth medium. As shown by Fig. 5, 1.0 μg/ml of poly(L-lys) markedly reduced and 2.0 μg/ml totally prevented the inhibitory effect of

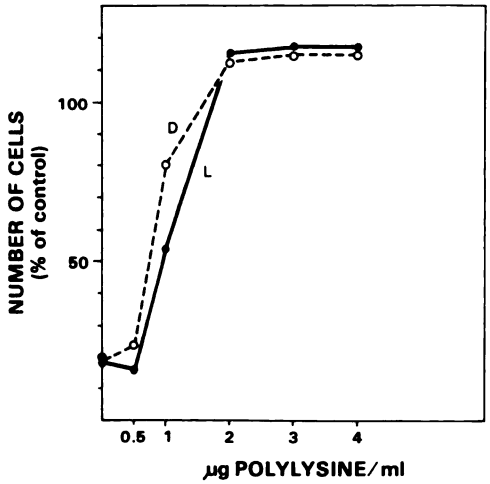

FIG. 5. *Growth inhibitory effect of MTX-poly(L-lys) in the presence of poly(L-lys) and poly(D-lys)*

Pro⁻3 Mtx^{RII} 5–3 cells were grown for 4 days in medium containing 1×10^{-7} M MTX-poly(L-lys) MW 70,000, in the presence of increasing amounts of either poly(L-lys) MW 70,000 (—●—) or poly(D-lys) MW 60,000 (----○----). Number of cells was counted after 4 day treatment and expressed as percent of controls grown without drug. The control flask contained 7.4 $\times 10^6$ cells.

MTX-poly(L-lys). The same qualitative and quantitative pattern of protection was observed when free poly(D-lys) was used instead of poly(L-lys). At high concentrations, poly(L-lys) appeared to increase the number of cells beyond the control values. This overshooting appears to be due to the fact that polylysine increases the adhesion of growing cells to their substrate, and thus slightly increases the number of cells per monolayer.

DISCUSSION

The observation that conjugation of MTX to poly(L-lys) drastically reduces its inhibitory effect on DHFR is interesting in view of the work of Whitehead (9), who showed that conjugation to hexaglutamate does not change the affinity of MTX for the enzyme. It is likely that the positive charges, rather than the size, contributed by the poly(L-lys) moiety prevent a normal binding of the conjugated MTX to DHFR. The finding that MTX-poly(L-lys) is an extremely poor inhibitor of dihydrofolate reductase *in vitro* implies that in order to inhibit cell growth, MTX-poly(L-lys) conjugate must be reactivated once ingested. On the basis of the data of Figs. 2, 3 and 4 we suggest that this reactivation is a result of the intracellular proteolytic breakdown of the poly(L-lys) carrier, followed by the release inside the cell of a small pharmacologically active adduct of MTX. Similarly we suggest that the lack of inhibitory effect of MTX-poly(D-lys) is due to the lack of breakdown of the poly(D-lys) carrier. We previously observed (3) that brief trypsinization of MTX-poly(L-lys) *in vitro* prior to its administration to cultured cells abolishes its inhibitory effect on transport-deficient cells, and conclude therefore that the digestion of the conjugate and the liberation of the pharmacologically-active MTX adduct must take place inside the cell and not in the tissue culture medium.

The route followed by the MTX-poly(L-lys) conjugate can be, to some extent, inferred from our prior observations with horseradish peroxidase-poly(L-lys) conjugation. We showed (1, 2) that reaction products of a horseradish peroxidase-poly(L-lys) conjugate are seen in numerous pinocytotic vesicles and vacuoles of various sizes, some of which share the morphology and localization of secondary lysosomes (digestive vacuoles). It is most likely, therefore, that the MTX-poly(L-lys) conjugates follow the same path, and that the enzymatic breakdown of the poly(L-lys) carrier occurs in secondary lysosomes. In order to inhibit cell growth the small molecular MTX adduct must be transported or must diffuse across the lysosomal membranes to reach its cellular targets. Since cells deficient in MTX transport are inhibited by MTX-poly(L-lys) it must be assumed that the passage of the MTX-adduct from the lysosome into the cytoplasm is not limited by the transport deficiency expressed at the cell surface. Several possible explanations can be suggested, among them an increased permeability of the lysosomal membrane, a different transport mechanism for the MTX adduct, or a high intralysosomal concentration of MTX-adduct. Warren, Nichols and Bender have shown that, at high concentrations of MTX, diffusion exceeds other forms of transport (10).

We showed that at a concentration of 1×10^{-7} M about 40 times more ^{3}H-MTX is taken up in one hour when given to transport proficient cells in the form of conjugate than when given in the form of a free drug (3). Yet Fig. 1 indicates that the biologic effect of MTX-poly(L-lys) is slightly less than that of free MTX. This discrepancy between transport and effect must be due in part to the fact that only a small fraction of the ingested conjugate is broken down inside the cell. In Fig. 3 this fraction is of the order of 25%. It is conceivable also that the small degradation product(s) contained in this fraction are pharmacologically less active than MTX. This question will be resolved when the breakdown product(s) are characterized and isolated in quantities that can be tested in the DHFR enzyme assay. It is not likely that MTX-poly-(glutamate) is a major constituent of this fraction, because the conversion of MTX to MTX-poly(glutamate) would not be complete under the conditions of our experiment (9), and free MTX would thus have to be detectable. It is most probable that the main degradation product(s) are MTX-lysine or MTX-oligolysines.

It is evident from these data that the intracellular release of pharmacologically-active MTX or MTX derivatives is a critical factor limiting the biological activity of MTX-poly(L-lys). The data of Fig. 5 show furthermore that this release process can be easily upset by experimental conditions such as the presence of competing amounts of poly(L-lys). It is noteworthy that although poly(D-lys) is not useful as a carrier for MTX it is as effective as poly(L-lys) in inhibiting the biologic effect of MTX-poly(L-lys) when present in excess in the growth medium. The mechanism of this inhibition is not yet understood. One attractive hypothesis is that only a fraction of the MTX-poly(L-lys) ingested is directed towards lysosomes. It is conceivable that the size of this fraction is concentration-dependent, and that an excess of poly(L-lys) decreases the fraction of MTX-poly(L-lys) that reaches lysosomes. It has been shown in another cell system that, in contrast to conventional ferritin, cationic ferritin is found not only in lysosomes, but also in stacked Golgi cisternae, in condensing vacuoles and in elements corresponding in description to GERL (11). It is conceivable that an excess of poly(L-lys) increases such a shift of transport away from secondary lysosomes.

Regardless of what fraction of MTX-poly(L-lys) may be reaching secondary lysosomes, this conjugate can be called a lysosomotropic drug (12, 13). Because the conjugate itself is not biologically active *in vitro*, it offers a good example of a "lysosome-activated drug." Since the specific activities of lysosomal proteolytic enzymes are often significantly higher in certain types of solid tumor than in their tissue of origin (14), it is conceivable that this drug conjugate may be found to be activated at a faster rate in some tumor tissues. Thus, besides overcoming transport resistance, MTX-poly(L-lys) may turn out to have some added selective toxicity toward tumor cells with high lysosomal activity. The effect of MTX-poly(L-lys) on tumor cells and tumor-bearing animals is now under investigation in our laboratory. Poly(L-lys) conjugated to 6-aminonicotinamide has been administered to tumor-bearing mice and found to have anti-tumor effects (15). This observation, as well as preliminary data from our laboratory, suggests a potential use of poly(L-lys)-drug conjugates as anti-tumor agents.

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REFERENCES

1. 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. Sciences (U.S.)* **75**: 1872–1876, 1978.
2. Ryser, H. J.-P., W.-C. Shen, and F. B. Merk. Membrane transport of macromolecules: New carrier functions of proteins and poly(amino acids). *Life Sci.* **22**: S13–15, 1253–1260, 1978.
3. Ryser, H. J.-P. and W.-C. Shen. Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cul-

tured cells. *Proc. Natl. Acad. Sciences (U.S.)* **75:** 3867–3870, 1978.

4. Shen, W.-C. and H. J.-P. Ryser. Conjugation of methotrexate to poly(L-lysine) and poly(D-lysine): Different inhibitory effects on cultured cells. *Fed. Proc.* **38,** part I, p. 258, 1979.
5. Stanley, B. G., G. E. Neal, and D. C. Williams. Dihydrofolate reductase (5, 6, 7, 8-tetrahydrofolate:NADP oxydoreductase, EC 1.5.1.3), in *Methods in Enzymology*, Vol. 18 (eds. S. P. Colowick and N. O. Kaplan). Academic Press, New York, 1971, 771–779.
6. Flintoff, W. F., S. F. Davidson, and L. Siminovitch. Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. *Somatic Cell Genet.* **2:** 245–261, 1976.
7. Flintoff, W. F. and L. Saya. The selection of wild-type revertants from methotrexate permeability mutants. *Somatic Cell Genet.* **4:** 143–156, 1978.
8. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell* **7:** 85–95, 1976.
9. Whitehead, V. M. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. *Cancer Res.* **37:**408–412, 1977.
10. Warren, R. D., A. P. Nichols, and R. A. Bender. Membrane transport of methotrexate in human lymphoblastoid cells. *Cancer Res.* **38:** 1668–1671, 1978.
11. Farquhar, M. G.: Recovery of surface membrane in anterior pituitary cells. Variation in traffic detected with anionic and cationic ferritin. *J. Cell. Biol.* **77:** R35–42, 1978.
12. DeDuve, C., B. DeBarsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoff. Lysosomotropic agents. *Biochem. Pharmacol.* **23:** 2495–2531, 1974.
13. Jacques, P. J. The selection and design of lysosomotropic drugs. *Adv. Exper. Med. Biol.* **73A:** 289–313, 1976.
14. Poole, A. R. Tumor lysosomal enzymes and invasive growth, in *Lysosomes in Biology and Pathology*, Vol. 3 (ed. J. T. Dingle). North-Holland/American Elsevier, Amsterdam, 1973, 303–337.
15. Arnold, L. J., Jr., A. Dagan, S. D. Simon and N. O. Kaplan. Polymer-linked derivatives of nicotinamide analogs: Elimination of toxic side effect with increase in anti-neoplastic activity. *Proc. Amer. Assoc. Cancer Res.* **19:** 114, 1978.