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# Tumor and Tissue Distribution of a Methotrexate–Anti-EL4 Immunoglobulin Conjugate in EL4 Lymphoma-bearing Mice[1]

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## ABSTRACT

The uptake and tissue distribution of [$^3$H]methotrexate ([$^3$H]MTX) at doses of 5 mg/kg i.p. either free or linked to anti-EL4 immunoglobulin G (AELG) or normal rabbit globulin (NRG) was studied in EL4 lymphoma-bearing C57BL/6J mice. When the uptakes of MTX-AELG, MTX-NRG, and free MTX were assayed as cell-associated $^3$H activity, comparison 3 hr after administration showed that uptake of MTX administered as the AELG conjugate was 2.5 times the uptake of MTX administered as the NRG conjugate and 6 times the uptake of MTX administered free. In contrast to the difference in the uptake of MTX-AELG and MTX-NRG by tumor cells, the pattern of uptake in all the other tissues studied was generally similar for the two conjugates. Conjugated MTX persisted in all tissues and serum and ascites fluid, whereas free MTX declined rapidly after reaching peak levels around 1 hr, except in EL4 cells where 45% was retained at 24 hr. The levels of intracellular MTX after administration of these three agents exceeded the intracellular dihydrofolate reductase level and correlated with the relative tumor-inhibitory effect *in vivo* of the agent (MTX-AELG > MTX-NRG > MTX).

## INTRODUCTION

MTX[4] covalently coupled to a rabbit IgG antibody against a tumor-associated antigen on the surface of mouse EL4 lymphoma cells was found to inhibit tumor growth more effectively *in vivo* than did free MTX, AELG alone, a mixture of MTX and AELG unlinked, or MTX linked to NRG (11). We have shown recently that when EL4 cells are exposed *in vitro* to free MTX or to MTX conjugated to either AELG or NRG, the cells take up more of the drug as the AELG conjugate than as the NRG conjugate or free MTX (14). We now report that more MTX was detected in tumor cells from mice given the MTX-AELG conjugate than in the tumor cells from mice given either the MTX-NRG conjugate or free MTX.

## MATERIALS AND METHODS

**Mice, Tumors, and Production of Rabbit AELG and MTX-Immunoglobulin Conjugates.** Female C57BL/6J mice (25 to 30 g; The Jackson Laboratory, Bar Harbor, ME) were inoculated i.p. with $10^7$ EL4 cells.

The details of the methods of production of rabbit AELG and of MTX-IgG conjugates have been described by us previously (11). In brief, antisera against the EL4 lymphoma were produced by repeatedly injecting adult New Zealand White rabbits with EL4 cells harvested from C57BL/6J mice into which the tumor had been inoculated 7 days before. The immune sera were rendered EL4 lymphoma specific by multiple absorptions with washed homogenates of liver, lungs, heart, kidney, and spleen from adult C57BL/6J mice. On immunofluorescence assay, these absorbed sera reacted only with EL4 cells and not with either cryostat sections of normal C57BL/6J mouse tissues or suspensions or smears of C57BL/6J lymphoid cells derived from lymph nodes, spleen, and thymus, B16 melanoma cells, 2 lines of AKR/J lymphoma cells, and Ehrlich ascites tumor cells. Sera were brought to 33% saturation by adding saturated aqueous ammonium sulfate. The precipitate was dissolved in PBS, and the ammonium sulfate was removed by repeated dialysis against PBS.

MTX was conjugated to IgG by the active ester intermediate method (11) using [$^3$H]MTX (37 Ci/mmol; New England Nuclear, Boston, MA) mixed with unlabeled MTX (Aldrich Chemical Co., Milwaukee, WI) to give a final specific activity of 2.5 mCi/mmol. Free MTX was separated from the MTX-IgG conjugate by column chromatography on Bio-Gel P100 and then by dialysis overnight against 1 liter of PBS. The average incorporation of MTX was 5 to 6 mol MTX/mol IgG. Repeat chromatography of this conjugate after it was stored for 1 week at 4° and pH 7.1 gave a single peak of radioactivity that eluted at the position of the conjugate in the initial chromatography. This shows that there was no dissociation of label from MTX or of the labeled MTX from the conjugate. Double dilution assay of $2 \times 10^7$ EL4 cells/ml, starting with MTX-AELG (1.0 mg/ml), failed to reveal any decrease in membrane immunofluorescence titer (0.025 mg/ml) compared to the parent AELG, *i.e.*, no decrease in antibody activity was detected after conjugation.

**Administration of Agents and Assay of Tritium in Tissues.** On the sixth day postinoculation, mice were given injections of [$^3$H]MTX (5 mg/kg i.p.) either free, conjugated to AELG, or conjugated to NRG, in a total volume of 1.0 ml of PBS. At various times thereafter, tumors were drained from the peritoneal cavity with a needle, the animals were killed by decapitation, their blood was collected, and several tissues were removed. Tumor cells were separated from ascites fluid by centrifugation at $600 \times g$ for 3 min and, after being washed 5 times with 15 ml of PBS, were dried to constant weight at 60°. The ascites fluid was air dried in Spectrapor dialysis tubing. Collected blood was allowed to clot at 37° and then was centrifuged; the resulting serum was air dried. Tissues were dried to constant weight at 60°.

For counting of radioactivity, samples were oxidized in an Oxymat sample oxidizer (Intertechnique, Paris, France). The isotopic recovery after oxidation was >95% with a memory of <0.05%. Blanks were oxidized between samples to eliminate this memory effect. Tritium was counted as $^3H_2O$ in an Oxifluor-$H_2O$ scintillation cocktail (New England Nuclear) in a Beckman LS 7000 scintillation counter (Beckman Instruments, Inc., Irvine, CA) with an efficiency of 37.5%.

**Efflux of Free MTX from EL4 Cells.** EL4 cells ($10^7$/ml) were incubated for 1 hr in serum-free RPMI Medium 1640 with either 1 $\mu$M [$^3$H]MTX (1.0 Ci/mmol) or 10 $\mu$M [$^3$H]MTX (0.16 Ci/mmol) in 35- x 10-mm tissue culture dishes at 37°. The cells were then washed 6 times with 10 ml of cold PBS after which they were incubated at 37° in 2 ml of medium without MTX. At various times, the cells were washed once with 10 ml of cold PBS then were dissolved in 2 ml of 0.01 M Tris-Cl buffer, pH 7.4, containing 0.1 M NaCl, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate

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[4] The abbreviations used are: MTX, methotrexate; AELG, anti-EL4 IgG; DHFR, dihydrofolate reductase; NRG, normal rabbit IgG; PBS, phosphate-buffered saline (0.01 M sodium phosphate, pH 7.1, containing 0.145 M sodium chloride).

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(10). Cell-associated radioactivity was determined in 1 ml of the lysate to which had been added 10 ml of Aquasol-2 (New England Nuclear).

## RESULTS

Chart 1 shows how free MTX, MTX-AELG, and MTX-NRG were distributed at various times after administration, in EL4 cells, liver, lungs, kidneys, brain, spleen, serum, and ascites fluid of tumor-bearing mice.

**Tissue Distribution of Free MTX.** The uptake of MTX reached peak levels in all tissues including serum at between 30 and 60 min. Peak levels were greatest in liver and kidney (18 to 20% of administered radioactivity per g, dry weight). Lung, spleen, and tumor took up lesser amounts (8.5, 5.5, and 4%, respectively), and the lowest uptake was in brain (0.25%). Tumor cells retained a substantial portion of the MTX taken up; approximately 45% of the peak value remained at 24 hr. MTX in ascites fluid declined steadily, reaching 0.8% of the administered dose per ml at 3 hr and 0.01% at 24 hr. After reaching its peak at 1 hr, the level in serum declined to 0.3%/ml at 3 hr and 0.03%/ml at 24 hr. In liver, spleen, kidney, and lungs, the major part of the accumulated MTX was cleared by 3 hr, and 1% or less remained at 24 hr. Although the peak level of uptake of MTX in brain was much lower than in tumor cells, its rate of clearance (judged by comparing radioactivities expressed as percentages of the peak activities) was slower than from any other normal tissue and was similar to that from EL4 cells, declining from 0.25% of the administered dose per g, dry weight, to 0.12% by 3 hr and remaining at that level at 24 hr.

**Tissue Distribution of MTX-AELG and MTX-NRG Conjugates.** The most striking finding was the difference in uptake between MTX-AELG and MTX-NRG by tumor cells (Chart 1). The amount of radioactivity in tumor cells from animals given the AELG conjugate increased progressively until, 3 hr after administration, those cells contained 15% of the administered dose per g, dry weight. This level was sustained for the rest of the 24-hr observation period. In contrast, the uptake of the MTX-NRG conjugate leveled off, at 1 hr, at 6% of the administered dose per g, dry weight. When the uptake of MTX-AELG, MTX-NRG, and free MTX were compared 3 hr after administration (*i.e.*, after reaching plateau levels), the level of MTX administered as the AELG conjugate was 2.5 times that of MTX administered as the NRG conjugate and 6 times that of MTX administered free (Chart 1).

In ascites fluid, substantial levels of both conjugates persisted, in contrast to the rapid clearance of free MTX (7 to 9% of the administered dose per ml compared to 0.01% at 24 hr for free MTX). Up to 3 hr, the proportion of the administered dose of MTX-AELG that remained in ascites fluid was slightly greater than that of MTX-NRG. Serum levels of both conjugates rose rapidly during the first hr, as did that of free MTX. As was true of the ascites fluid levels, throughout the 24-hr period of observations the serum levels of mice that received one of the conjugates were higher than those of mice that received free MTX.

In contrast to the difference in the uptake of MTX-AELG and MTX-NRG by tumor cells, the pattern of uptake of the 2 conjugates was similar in all the other tissues studied, with the possible exception of brain. Brains of mice given MTX-AELG showed a somewhat higher MTX uptake at 24 hr than did those of mice given MTX-NRG, but this tissue took up much lower levels of MTX, free or conjugated, than any other tissue analyzed. The rate of uptake of conjugated MTX by liver, kidney, and lungs was slower than that of free MTX, and the extent of uptake was less than the peak value attained by free MTX at 1 hr. In all tissues, conjugated MTX persisted throughout the 24-hr observation period, unlike free MTX which was cleared very quickly (except perhaps in brain). In spleen and brain, the extent of uptake of conjugates exceeded that of free MTX, and these higher levels also persisted.

Measuring efflux of MTX from cells *in vitro* (12) provides an estimate of the intracellular level of DHFR based on the high-affinity stoichiometric binding of MTX to DHFR. When MTX was allowed to efflux from MTX-loaded cells by incubating those cells in MTX-free medium, the concentration of intracellular MTX leveled off at 5 pmol/mg protein (Chart 2, *inset*). When the results of uptake of the 3 agents were expressed as pmol of MTX per mg of EL4 cell protein, it is obvious that all uptake levels exceeded intracellular DHFR (Chart 2).

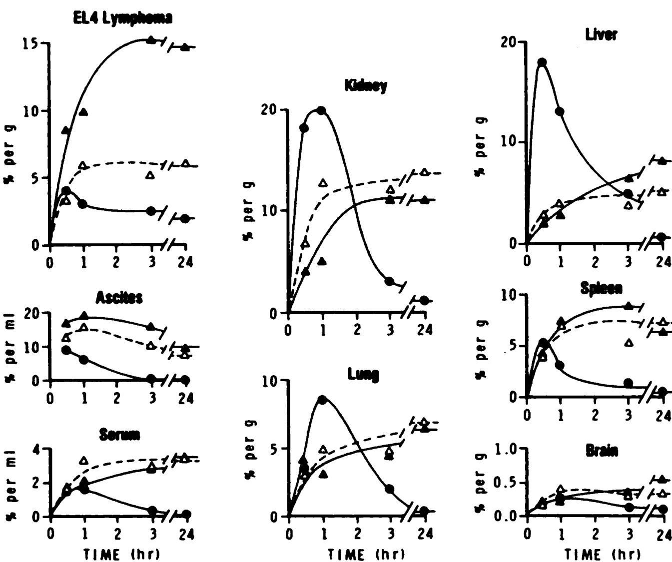


Chart 1. Uptake and clearance of free MTX, MTX-AELG, and MTX-NRG in ascites fluid, serum, and tissues of EL4 lymphoma-bearing mice. Mice were inoculated i.p. with $10^7$ EL4 cells and, 6 days later, were given injections of a 5-mg/kg dose i.p. of either free $[^3H]$MTX (●), $[^3H]$MTX-AELG (▲), or $[^3H]$MTX-NRG (△), in a total volume of 1.0 ml of PBS. At the times indicated, tissue samples were obtained and were counted for tritium as described in "Materials and Methods." Tissue-associated radioactivity is expressed as the percentage of the administered dose per g, dry weight, of tissue (%/g) or for ascites fluid and serum, per ml (%/ml). Each value is the mean of determinations on 3 samples from different animals.

## DISCUSSION

We have investigated the mechanism of the superior tumor inhibition by MTX-AELG conjugates compared to that of free MTX or MTX conjugated to NRG that has been demonstrated by us in EL4 lymphoma-inoculated mice (11). The results reported here show that EL4 cells in tumor-bearing animals took up the greatest amount of MTX when injected with this drug in the form of its AELG conjugate. These findings extend our earlier *in vitro* investigations (14) in which we incubated EL4 cells with free MTX and MTX conjugates at 0° and 37° and found that uptake was greatest for the AELG-linked drug. Also in keeping with our earlier *in vitro* results, administration of MTX as the NRG conjugate to tumor-bearing animals led to less uptake of drug by tumor cells than did its administration as the AELG conjugate but more than it did as the free drug (14).

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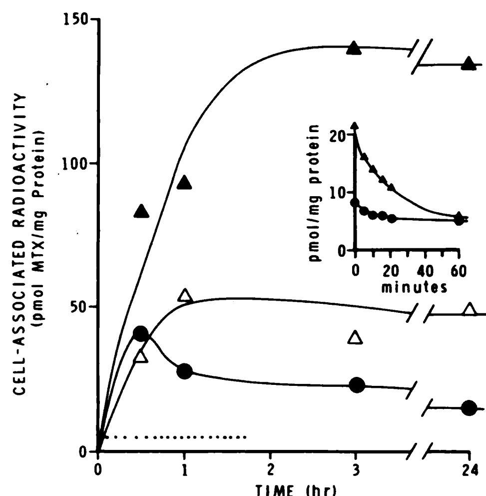


Chart 2. Uptake of free MTX (●), MTX-AELG (▲), and MTX-NRG (△) by tumor cells in EL4 lymphoma-bearing mice. The experimental conditions were as specified in Chart 1, but the results of determination of cell-associated radioactivity are expressed as pmol of MTX per mg protein. ····, DHFR level in these cells, estimated from the data for efflux shown in the *inset*. *Inset*, efflux of MTX from EL4 lymphoma cells *in vitro*. Cells ($10^7$/ml) in serum-free RPMI medium were incubated for 1 hr with [$^3$H]MTX at a concentration of either 1.0 (●) or 10 $\mu$M (▲), after which they were washed and transferred to MTX-free medium. At the times indicated, radioactivity remaining associated with the cells was determined as described in "Materials and Methods."

Following the i.p. injection of MTX and its conjugates into ascites tumor-bearing mice, absorption from ascites fluid into blood and other tissues was rapid, peak levels being reached by the free drug within 1 hr and by either conjugate within approximately 3 hr. Conjugates were cleared more slowly from ascites fluid than was free MTX; both exhibited slightly higher levels at 1 hr than at 30 min, whereas the free drug showed progressive clearance from the time of the first measurement. Since samples of ascites fluid were collected from the side of the abdomen opposite to the site of administration, diffusion of conjugates in this viscous fluid may have been slower than that of free MTX. After 1 hr, both conjugates declined, but the clearance of MTX-AELG was somewhat slower than that of MTX-NRG. This may have been due to binding of AELG to antigen either on the EL4 cell surface or free in the ascites fluid, which would also explain the slightly lower levels fo MTX-AELG in serum compared to MTX-NRG (Chart 1). However, the higher level of MTX-AELG in EL4 cells was not due simply to its higher level in ascites fluid. The superior carrier ability of AELG compared to NRG could be seen, *e.g.*, from the data obtained at 3 hr showing that tumor cells took up 3.0 times as much MTX conjugated to AELG as MTX conjugated to NRG, even though the ratio in the ascites fluid was only 1.6 (Chart 1). The corresponding figures for 24 hr were 2.5 and 1.4, respectively.

In most tissues, free MTX reached a peak by 1 hr, then declined steadily to low levels. This decline was most pronounced in liver and kidney, where the initial peak had been almost 20% of the administered dose per g, dry weight. In tumor cells, the initial peak was 4% of the administered dose per g, dry weight, and the rate of decline was slower than in other tissues; at 1, 3, and 24 hr, tumor cells contained approximately 1.2, 1.0, and 0.6 nmol MTX/g, wet weight, respectively. The corresponding figures for ascites fluid were 21, 2.5, and 0.04 nmol/ml, and those for serum were 5.3, 1.1, and 0.1 nmol/ml. Others have also reported that MTX-sensitive tumors clear MTX activity more slowly than do other tissues (1, 2, 4, 9).

Uptake of MTX linked to NRG by the EL4 cells has been attributed to the high pinocytotic activity of those cells (3, 6), and pinocytosis could also play a role in uptake of the AELG conjugate. However, an additional, more important uptake mechanism for MTX-AELG is probably capping and endocytosis at 37° subsequent to binding of the AELG conjugate to specific receptors on the cell surface, as we have shown by *in vitro* experiments at 0° and 37° with AELG carrying MTX (14) or chlorambucil (8). The fact that the level of immunoglobulin-linked MTX in ascites fluid and serum was persistently higher than the level of free MTX could also have contributed to higher initial uptake and persistence of high levels of MTX in the tumor cells.

Studies with free MTX have shown that cytotoxic action correlates with the excess of drug over the stoichiometric DHFR level and the duration of exposure (7). Maintenance of such high levels is facilitated by formation of polyglutamates that are retained better by the cell than is MTX itself (5, 7). Our efflux studies gave a value for the DHFR level in these tumor cells of approximately 0.3 nmol/g, wet weight (Chart 2); therefore, administering 5 mg free MTX/kg could maintain cytotoxic amounts for at least 24 hr. The more effective tumor-inhibitory action that we reported before (11) for both MTX-AELG and MTX-NRG at this dose level may be due to the higher levels of intracellular drug now demonstrated to persist during our observation period of 24 hr. Another contributory factor could be sustained release of more potent free MTX by intracellular catabolism of the conjugate. Incubation of MTX-NRG with an EL4 homogenate produced a low-molecular-weight [$^3$H]MTX-containing fragment but no free MTX (14). Our recent studies on this and other similar MTX-containing catabolic products derived from MTX-IgG conjugates have not shown them to be more effective inhibitors of DHFR *in vitro* than the parent conjugates,[5] which are themselves approximately half as inhibitory as free MTX (11). The loss of potency of conjugated MTX could have been more than compensated for by the large excess of drug persisting in EL4 cells 24 hr after administration as a conjugate (*e.g.*, 135 pmol MTX/mg protein in mice given MTX-AELG compared to 17 pmol MTX/mg in mice given free MTX). Measurements beyond 24 hr would have shown how long the excess (relative to the level of intracellular DHFR) produced by conjugate administration could be maintained in comparison with the level produced by free MTX administration. Unfortunately, our EL4 lymphoma model has not allowed a longer period of observation. An interval of 7 days between i.p. inoculation of $10^7$ EL4 cells and sacrifice was necessary for the development of the ascites tumor, and after 7 days, the ascites fluid contained substantial numbers of RBC (14) that also take up MTX (13). Furthermore, tumor-inoculated mice started dying after the eighth day. However, our results indicate that the higher plateau levels of the drug administered as the conjugate are likely to be maintained well beyond 24 hr.

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[5] P. Uadia, A. H. Blair, and T. Ghose. Uptake of MTX linked to poly- and monoclonal anti-melanoma antibodies by a human melanoma line, submitted for publication to J. Nat. Cancer Inst.



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