MOLECULAR PHARMACOLOGY, 13, 80-88

High Molecular Weight Derivatives of Methotrexate as Chemotherapeutic Agents

BARBARA C. F. CHU AND JOHN M. WHITELEY[1]

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

(Received March 29, 1976)
(Accepted August 30, 1976)

SUMMARY

BARBARA CHU, C. F. & WHITELEY, JOHN M. (1977) High molecular weight derivatives of methotrexate as chemotherapeutic agents. *Mol. Pharmacol.*, 13, 80-88.

Female BDF_1 mice treated with methotrexate (MTX) covalently bound to bovine or murine serum albumin (BSA, MSA) show a higher, more prolonged serum concentration and a decreased rate of excretion of MTX compared with a similar group of mice treated with free MTX. Tritiated MTX-albumin derivatives circulate to the tissues as the covalent complexes but are cleaved prior to excretion and exit primarily as unbound MTX. [^{3}H]MTX-albumin derivatives injected intraperitoneally into L1210 tumor-bearing mice results in prolonged localization in the ascitic fluid and elevated intracellular MTX levels after 24 hr. Approximately 90% of the tritium label found in the L1210 cells is located in the cell lysate as free, unmetabolized MTX, whereas when the albumin carrier is labeled with ^{125}I, 80% of the radioactivity is found associated with the cell membrane. A single dose of MTX-BSA (equivalent to 15 mg of MTX per kilogram) injected into BDF_1 mice 24 hr after inoculation of 10^6 L1210 cells is as effective as MTX in prolonging survival time from 8 (control) to 15 days. An equivalent dose of MTX-MSA, however, shows considerable toxicity. MTX-aminoethyldextran derivatives of mol wt 10,000-150,000 are ineffective antitumor agents. These observations suggest that the high molecular weight MTX-albumin derivatives are retained in the serum and extracellular compartments until the complexes are hydrolyzed, and thus markedly increase the lifetime of MTX within the animal.

INTRODUCTION

Although there have been numerous attempts to improve the chemotherapeutic properties of methotrexate by altering its molecular structure (1-4), none of the derivatives yet described has demonstrated sufficiently different biological properties to warrant its continual use in therapy as a replacement for the original antimetabolite. In most of these cases the diamino-substituted pyrimidine portion of the MTX[2] structure has been retained, and heteroatoms have been either inserted into or omitted from the pyrazine ring or aromatic side chain of the drug, leaving a molecule which possesses physicochemical properties similar to MTX itself. It is possible, however, that if MTX could be modi-

This investigation was supported by grants from the National Cancer Institute (CA-11778) and National Institute of Arthritis and Metabolic Diseases (AM-07097) and by Grant BC-62M from the American Cancer Society.

[1] Recipient of Research Career Development Award CA-00106 from the National Cancer Institute; to whom requests for reprints should be addressed.

[2] The abbreviations used are MTX, methotrexate; BSA, bovine serum albumin; MSA, murine serum albumin.

fied by substitution into a polymeric compound of much greater molecular weight, the resultant derivative would retain many of the properties of this larger molecule and therefore might (a) be retained within the body and, by slow hydrolysis, release a constant high level of MTX which could be therapeutically beneficial in the treatment of systemic lesions, and (b) if the carrier were of sufficiently high molecular weight, be restricted to a body cavity containing a solid tumor, and thus offer antagonistic properties not usually observed with free MTX because of its rapid excretion via the renal and circulatory systems.

We have previously described the covalent attachment of MTX to soluble starch to give an affinity matrix which aided the purification of dihydrofolate reductase from chicken liver (5). The present investigation describes the preparation and purification of similar compounds obtained by linking MTX covalently to BSA, MSA, and various high molecular weight dextrans. The chemical properties of these high molecular weight derivatives of MTX are reported, together with a preliminary examination of their biological behavior in BDF_1 mice. Observations of how long and in what form the derivatives persist in the blood, how they are excreted, and how toxic they may be are presented, and, as a prelude to investigating their behavior in the therapy of solid tumors or MTX-resistant cell lines, their effect on the well-defined murine L1210 ascites tumor (6, 7) has been determined.

MATERIALS AND METHODS

Materials. The following were obtained from commercial sources: Bio-Gel P-100, 100–200 mesh, Bio-Rad Laboratories; DE-52 cellulose, Whatman Biochemicals; Aquasol, 2,5-diphenyloxazole, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, New England Nuclear Corporation; crystalline BSA, Sigma Chemical Company; 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride, Story Chemicals; and [3′,5′-^{3}H]MTX, 10 Ci/mmole, Dhom Products, Los Angeles. The ^{3}H-labeled MTX was 96–98% pure upon paper chromatography in system A (see below). MTX and 4-amino-4-deoxy-10-methylpteroic acid were gifts from Dr. Florence White, Cancer Chemotherapy National Service Center, National Cancer Institute, and from the Children's Cancer Research Foundation, Boston, respectively. Tris-HCl buffer solutions were prepared by titrating aqueous Tris (Sigma) with hydrochloric acid at ambient temperature. Before use at 0°, the pH of the solution was corrected for changes caused by pK variance with temperature.

Dextrans T10, T40, T70, and T150 (Pharmacia) were converted to their aminoethyl derivatives by base-catalyzed condensation with 2-aminoethyl hydrogen sulfate (Eastman Kodak; recrystallized from 65% aqueous ethanol before use) according to Semenza (8). The reaction required 2.5 hr at 95°, and the product was isolated by dialysis against water (48 hr) and lyophilization. *N*-Methyl-*p*-aminobenzoic acid and *N*-methyl-*p*-aminobenzoylglutamic acid were synthesized according to Cosulich and Smith (9).

The L1210 cell line (established in DBA_2 male mice), obtained from Arthur D. Little, Inc., Cambridge, Ma., was maintained in continuous culture for 8 months and then preserved at −190°. Before use in these experiments, the revived cell line was re-established in DBA_2 male mice. Female BDF_1 mice (19–22 g; Simonsen Laboratories, Gilroy, Ca.) were used for all experiments reported in this investigation.

Methods. Analytical chromatography on Whatman No. 1 paper used the following solvent systems: A, methanol–ammonia–water (7:1:2, v/v); B, ethanol–water (4:1, v/v); C, 0.1 M potassium phosphate buffer, pH 7.0. System A was descending; the latter two were ascending. Radioactivity of all aqueous samples of tritiated material was measured in a Beckman LS-233 liquid scintillation counter; 1-ml samples were mixed with Aquasol (10 ml) before counting. Chromatography papers containing radioactive compounds were cut into 2 × 4 cm strips, then suspended in counting vials containing 16 ml of a scintillant prepared from 2,5-diphenyloxazole (6 g) and 1,4-bis[2-(4-methyl-5-phenyloxa-

zolyl)]benzene (0.5 g) in toluene (1 liter) prior to measurement. ^{125}I-Containing samples were counted in a Nuclear-Chicago 1185 γ-counter.

MSA was isolated by the procedure outlined for the purification of rabbit serum albumin by Sonoda and Schlamowitz (10). Anesthetized, 6-week-old BDF_1 mice (104 total) were bled via eye excision; from 95 ml of whole blood, 43 ml of serum were obtained. The volume was adjusted to 50 ml with 0.2 M phosphate buffer, pH 8, and globulins were precipitated by the addition of solid ammonium sulfate to 50% saturation. Centrifugation afforded a clear supernatant fraction, from which MSA was precipitated with ammonium sulfate at 90% saturation. Further purification was achieved by extracting the precipitate three times with a 60% saturated solution of ammonium sulfate, followed by dialysis for 48 hr against 0.02 M phosphate, pH 8, and chromatography on DEAE-cellulose. Elution was accomplished using a linear gradient of NaCl (0–0.5 M) in 0.02 M phosphate buffer, pH 8.0. MSA was the major emergent peak. A total of 0.96 g of albumin, essentially homogeneous by polyacrylamide gel electrophoresis, with an R_F of 0.65, was recovered. In some preparations a minor contaminant at R_F 0.40 was observed.

MTX-BSA was synthesized by the simultaneous addition of MTX (40 mg, 0.09 mmole) and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (25 mg, 0.13 mmole), each dissolved in 2 ml of 0.05 M sodium bicarbonate buffer, pH 7.6, to 100 mg of BSA dissolved in 7 ml of the same buffer. The reaction mixture was clarified by gentle shaking, then maintained at room temperature in the dark for 4 hr. The products were applied to a 2.2 × 30 cm column of Bio-Gel P-100 and eluted with 0.1 M potassium borate, pH 8.2, containing 1 M urea. Collection of 5-ml fractions afforded MTX derivatives in fractions 12–25 and unreacted MTX in fractions 38–56. Fractions 15–25 were pooled, dialyzed against H_2O for 24 hr, and lyophilized. The results of several preparations showed the MTX content to be 75–86 mg/g of albumin, calculated from the absorbance at 370 nm in 0.1 N NaOH (11). Absorbance properties of MTX-BSA in 0.1 N NaOH were similar to those previously reported, with an A_{370}:A_{300} ratio of 0.3 (12). The MTX derivatives of MSA and aminoethyldextrans T10, T40, T70, and T150 were prepared by similar procedures.

[^{125}I]MTX-BSA and [^{125}I]MTX-MSA with a specific activity of 1.5 μCi/mg were synthesized from the nonradioactive MTX-albumin derivatives by the procedure of McConahey and Dixon (13) with the kind cooperation of Mr. Frank Tooker, Department of Immunopathology, Scripps Clinic and Research Foundation. The absorbance properties of [^{125}I]MTX-BSA (λ_{max} = 255, 303, and 370 nm in 0.1 N NaOH) were identical with those of noniodinated MTX-BSA. This observation confirmed that iodination had occurred only in the protein molecule, as the 3′,5′-halogenated derivatives of MTX have no absorption maximum at 303 nm (14).

For determinations of plasma drug levels and excreted metabolites, BDF_1 mice were injected intraperitoneally with 7.5 mg/kg of [^{3}H]MTX-BSA, [^{3}H]MTX-MSA, and [^{3}H]MTX (specific activities, 650 μCi of MTX per millimole) or with 2.5 mg/kg of [^{125}I]MTX-BSA (1.5 μCi/mg).[3] For the measurement of plasma drug levels, blood samples (30 μl) were removed from the eye with heparinized blood-collecting microtubes 0, 0.12, 0.25, 0.5, 1, 2, 4, 7.5, and 24 hr after injection. Experiments in which uncomplexed [^{3}H]MTX was injected were terminated after 1 hr, as the blood levels were no longer measurable. Erythrocytes were separated by centrifugation for 5 min in a Beckman 152 Microfuge, and the radioactivity in a 20-μl aliquot of the plasma was measured. Larger samples were mixed with nonradioactive MTX as a carrier and were analyzed by chromatography in system A. MTX-albumin remained at the origin, whereas MTX migrated with an R_F of 0.66. For measurement of excreted metabolites, urine was collected 8,

[3] All dosages reported for the high molecular weight derivatives of MTX refer to the quantity of MTX contained by the complex per kilogram of mouse body weight.

24, and 48 hr after injection; feces were collected after 24 and 48 hr. Feces (approximately 0.15 g), frozen overnight to lyse intact cells and suspended in 5 ml of 0.05 M phosphate buffer, pH 7, were homogenized with the aid of an automatic mixer and then heated for 5 min in a boiling water bath. Samples were cooled and centrifuged, and 0.5-ml aliquots were monitored for radioactivity. Radioactivity measurements were made on urine samples (0.1 ml) without further processing. For identification of excreted components, larger urine samples were lyophilized, mixed with nonradioactive MTX, and analyzed by sequential paper chromatography in solvent systems A, B, and C with the use of known standards.

For studies of drug transport into L1210 cells, BDF_1 mice were injected, on the sixth or seventh day after inoculation of 10^6 L1210 cells, with 15 mg/kg of [^{3}H]MTX-BSA, [^{3}H]MTX-MSA, [^{3}H]MTX, or [^{3}H]MTX-dextran T70 (specific activities, 650 μCi/mmole) or with 3.5 mg/kg of [^{125}I]MTX-BSA (1.5 μCi/mg). One hour after injection, ascitic fluid (1.0–1.2 ml) was removed from the mice without killing the animals and diluted with 10 ml of 0.01 M Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl and 0.1 mg/ml of EDTA. Twenty-four hours after injection the animals were killed and the remaining fluid (approximately 1.0–1.2 ml) was removed and diluted with buffer. Throughout these experiments cold (approximately 0°) buffer solutions were used to reduce the possibility of drug leakage from the cells. The total number of harvested cells was determined in a hemocytometer, and the following sequence of operations was performed. (a) The cell suspension was sedimented by centrifugation (8 min, International clinical centrifuge) and washed with 5 ml of buffer, and an aliquot of the combined supernatant fractions was measured for radioactivity to determine the quantity of activity in the extracellular fluid (see Table 3). (b) Erythrocytes in the cell pellet were lysed by suspension of the cells in 0.035 M NaCl for 30 sec. Isotonicity (0.15 M) was restored by the addition of concentrated NaCl, and the remaining intact ascites cells, sedimented by centrifugation, were washed with Tris-HCl buffer, pH 7.2, containing 0.065 M NaCl, 0.015 M KCl, and 0.008 M $CaCl_2$, until no more radioactivity was found in the washings. (c) The cells were resuspended in 3 ml of the same 0.01 M Tris-HCl buffer, and, after a 1:50-diluted sample of this suspension had been counted in a hemocytometer, 1 ml was monitored for radioactivity. The cells were then lysed by freezing and thawing. After centrifugation, measurement of the radioactivity of the lysate and the membrane precipitate indicated the quantity of free and membrane-associated antagonist in the sample.

In order to determine how much radioactivity leached out of the cells during the osmotic shock treatment in step (b), a separate experiment was performed. After the cells had been harvested, they were washed with buffer until no more radioactivity was found in the wash fluid. A portion of the cell suspension was monitored for radioactivity before the hypotonic shock treatment. The cells were then sedimented, suspended in hypotonic NaCl for 30 sec, and returned to isotonicity with concentrated NaCl. After centrifugation and resuspension in fresh buffer, a portion of this was monitored for radioactivity. Results from three experiments indicated that between 0% and 10% of the radioactivity had leached out during the hypotonic NaCl treatment.

MTX and its derivatives were tested for antitumor activity using the protocol recommended by the Drug Evaluation Branch, National Cancer Institute (15). Mice were inoculated with 10^6 L1210 cells on day 0, and the drug was injected intraperitoneally on day 1. Tabulated results are expressed as mean values ± standard deviations.

RESULTS

Synthesis of high molecular weight derivatives of MTX. These compounds were prepared by the carbodiimide-promoted condensation of aminoalkyldextrans or albumins with MTX according to a procedure originally described by Mell (5). During the albumin coupling reactions it was

noted that covalent linkage between polymers increased when the concentration of carbodiimide greatly exceeded that of MTX; even with equivalent concentrations, some aggregation occurred. However, during chromatography on Bio-Gel P-100, the aggregated components were concentrated in the first three fractions of the product peak. They were identified by a lower R_F value and different band pattern on polyacrylamide electrophoresis and were discarded. The desired product fractions had electrophoretic profiles identical with those of the starting materials.

Determination of plasma drug levels. Figure 1 illustrates the levels of [^{3}H]MTX, [^{3}H]MTX-BSA or -MSA, and [^{125}I]MTX-BSA found in the plasma at different time intervals after intraperitoneal injection of the indicated derivative (dose, 7.5 mg/kg for each tritiated sample; 2.5 mg/kg for the ^{125}I-containing complex). MTX reached a maximum concentration of 7 μg/ml in 5–10 min, and after 1 hr this level had dropped to 1 μg/ml. In contrast, both MTX-BSA and MTX-MSA reached their maximum concentration in the plasma after 1–2 hr and then decreased steadily; at 24 hr the concentration had dropped to 1–2 μg/ml. A maximum concentration of 35–36 μg/ml was achieved, 5 times higher than with nonderivatized MTX. The percentages of injected radioactivity per milliliter of plasma found for [^{3}H]MTX-BSA, [^{3}H]MTX-MSA, and [^{125}I]MTX-BSA between 0 and 7 hr were very similar, although the tritium-labeled component disappeared at a slightly higher rate than its ^{125}I-labeled carrier molecule. The close resemblance between the two curves suggests that MTX remains covalently bound to the albumin carrier when circulating in the plasma. This conclusion is supported by the observation that when serum samples (0.1–0.15 ml) from [^{3}H]MTX-BSA-treated mice, taken after 2 and 7 hr, were analyzed by chromatography in system A, 92–98% of the radioactivity remained at the origin, as did the MTX-BSA marker. When plasma samples of [^{3}H]MTX-treated mice were analyzed by chromatography in the same way, all the radioactivity migrated with the MTX marker at R_F 0.66.

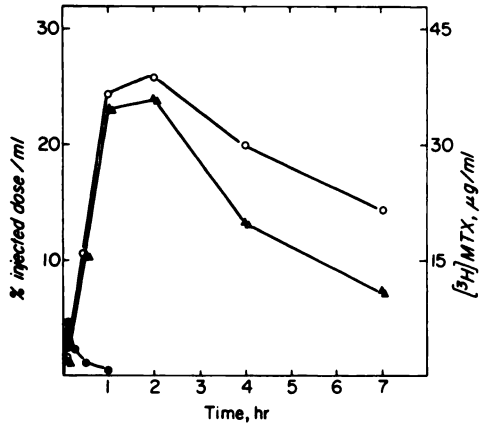

FIG. 1. *Time-dependent variation of plasma drug concentrations found in BDF$_1$ mice treated with high molecular weight MTX derivatives*

The left ordinate shows the time-dependent variation of the percentage of injected radioactivity, and the right ordinate shows the concentration of MTX found per milliliter of plasma in BDF$_1$ mice after intraperitoneal injection of 7.5 mg/kg of [^{3}H]MTX (●), [^{3}H]MTX-BSA (▲), or [^{3}H]MTX-MSA (△) (specific activity, 650 μCi/mmole) or 2.5 mg/kg of [^{125}I]MTX-BSA (○) (1.5 μCi/mg).

Analysis of excreted metabolites. Table 1 shows that when unbound [^{3}H]MTX (7.5 mg/kg) was injected intraperitoneally into BDF$_1$ mice, more than 50% of the label was excreted in the urine after 8 hr, and 77–93% was recovered from both urine and feces after 24 hr. In contrast, only 16–26% was excreted in the urine 8 hr after a similar dosage of [^{3}H]MTX-BSA or -MSA, and 52–65% was recovered after 24 hr. Even after 48 hr the total recovery of the derivatized MTX was only 56–75%, emphasizing the slow rate of drug release in this instance. Similarly, only 58–66% of the ^{125}I label was recovered 24 hr after injection of [^{125}I]MTX-BSA or -MSA.

When urine samples from both [^{3}H]-MTX- and [^{3}H]MTX-BSA-treated animals were analyzed, each contained identical metabolites. After chromatography twice in system A to purify these metabolites, three discrete radioactive zones were found. The component with R_F 0.66 corresponded to MTX and contained 73–80% of the total radioactivity. Two other radioactive zones, with R_F 0.56 and 0.82, containing 3–6% and 13–17%, respectively,

TABLE 1

Excretion of radioactively labeled, MTX-containing drugs injected into BDF$_1$ mice

Each BDF$_1$ mouse received an intraperitoneal injection of 7.5 mg/kg of [^{3}H]MTX or [^{3}H]MTX-BSA (-MSA) (specific activity, 650 μCi/mmole) or 2.5 mg/kg of [^{125}I]MTX-BSA (1.5 μCi/mg). Values are the range of percentages obtained by treating groups of four mice with each of the drugs.

Drug	Urine			Feces		Total	
	8 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
	%	%	%	%	%	%	%
[^{3}H]MTX	53–57	67–72	69–76	10–21	11–23	77–93	80–96
[^{3}H]MTX BSA or -MSA	16–26	45–55	49–61	5–10	7–14	52–65	56–75
[^{125}I]MTX-BSA or -MSA		55–60	59–65	3–6	4–8	58–66	63–73

of the total radioactivity, corresponded to 4-amino-4-deoxy-10-methylpteroate and either *N*-methyl-*p*-aminobenzoate or *N*-methyl-*p*-aminobenzoylglutamate. Rechromatography in systems B and C confirmed that the third component was the glutamate derivative. The main radioactive product (94%) isolated from the urine of mice treated with [^{125}I]MTX-BSA was not the intact BSA molecule, but a product which had the same R_F as both iodotyrosine and diiodotyrosine in system A.

Toxicity and antineoplastic effects of high molecular weight derivatives of MTX against L1210 tumors. At a dose of 56.5 mg/kg, 4 times the therapeutic level, MTX-treated, non-tumor-bearing mice showed a 1.5-g weight loss after 1 day, but gradually regained this weight and survived for 30 days, when the experiment was terminated. At the same dosage, MTX-BSA-treated mice lost an average of 2 g after the first day and a further 1 g each successive day, until the end of day 5, when they died of toxicity, weighing approximately 15 g. Non-tumor-bearing mice treated with only 16 mg/kg of MTX-MSA all died of toxicity within 5 days, although at 15-mg/kg doses they lost an average of 5 g and 60% gradually regained this weight and survived for 30 days. Mice treated the same way with BSA or MSA alone showed no weight loss or toxicity after 30 days.

MTX-BSA, -MSA, and -dextrans were compared with MTX as antitumor agents against L1210 leukemic cells in BDF$_1$ mice (Table 2). In each experiment a single dose of 15 mg/kg was injected intraperitoneally 24 hr after the animal had been inoculated with 10^6 L1210 cells. With this treatment

TABLE 2

Survival times of L1210 tumor-carrying BDF$_1$ mice treated with high molecular weight derivatives of MTX

BDF$_1$ mice were inoculated on day 0 with 10^6 L1210 cells and treated on day 1 with either MTX or a series of high molecular weight derivatives of MTX.

Antagonist	Quantity administered as MTX	No. of mice	Mean survival time
	mg/kg		*days*
None (control)	0	44	8.0 ± 1.2
MTX	15	13	15.0 ± 2.3
MTX-BSA	15	8	14.7 ± 1.7
MTX-MSA	15	11	14.3 ± 2.6[a]
MTX–dextran 10	15	14	10.1 ± 2.6
MTX–dextran 40	15	11	9.5 ± 2.3
MTX–dextran 70	15	6	8.7 ± 1.3
MTX–dextran 150	15	6	10.3 ± 4.0

[a] In this experiment seven mice died of drug toxicity, and are excluded from the survival figure.

schedule MTX-BSA was equally as effective as free MTX, whereas MTX-MSA was comparatively toxic (7 out of 11 mice died) and the MTX-dextrans were ineffective.

Determination of intra- and extracellular [^{3}H]MTX. Measurements were made of the amount of [^{3}H]MTX found both in the peritoneal extracellular ascitic fluid and within the L1210 cells 1 and 24 hr after an intraperitoneal injection of 15 mg/kg of [^{3}H]MTX or [^{3}H]MTX-carrier (Table 3). After 1 hr a concentration of 15.2 μg/ml, or a total weight of 30.7 μg (10.2% of the injected radioactivity), was observed in the extracellular ascitic fluid of [^{3}H]MTX-treated mice. During the same period 40–

TABLE 3

Intracellular (MTX) and extracellular (MTX or MTX-carrier) peritoneal drug concentrations exhibited by L1210 tumor-infected mice

BDF_1 mice were inoculated on day 0 with 10^6 tumor cells and on day 6 with either tritiated MTX, MTX-BSA, MTX-MSA, or MTX-dextran T70 (15 mg/kg corresponds to approximately 300 μg/animal). The MTX levels were measured 1 and 24 hr after drug inoculation. A minimum of six mice were used to determine each value.

Sample	Extracellular		Intracellular	
	1 hr	24 hr	1 hr	24 hr
	μg/ml		*μg/10^8 L1210 cells*	
[^{3}H]MTX	15.2 ± 3.75	0.55 ± 0.05	0.20 ± 0.04	0.075 ± 0.02
[^{3}H]MTX-BSA	51.4 ± 9.3	8.8 ± 1.9	0.07 ± 0.01	0.10 ± 0.02[a]
[^{3}H]MTX-MSA	55.6 ± 4.9	12.1 ± 1.5	0.22 ± 0.04[a]	0.15 ± 0.04[a]
[^{3}H]MTX-dextran T70	39.7 ± 8.6	3.9 ± 0.9	0.09 ± 0.01	0.08 ± 0.02[a]

[a] Not statistically significant relative to the MTX values ($p < 0.05$).

56 μg/ml, or a total of 115–133 μg (38–44%), were found in the mice treated with MTX-albumin or MTX-dextran. After 24 hr the concentration of drug left in the extracellular fluid after treatment with free [^{3}H]MTX had dropped to 0.55 μg/ml, a total weight of 0.96 μg or 0.32% of the injected radioactivity. In contrast, a concentration of 4–12 μg/ml (10–17 μg,3–6%) persisted in the fluid of the mice treated with a ^{3}H-labeled, high molecular weight derivative of MTX. Chromatography of a portion of the 1-hr ascitic fluid from [^{3}H]MTX-carrier-treated mice in system A showed that the MTX remained bound to its carrier.

More than half the label present in the cells of [^{3}H]MTX-treated mice after 1 hr (0.20 μg/10^8 cells) migrated from the cells within 24 hr. In the animals treated with [^{3}H]MTX-BSA and [^{3}H]MTX-dextran T70, the amount of [^{3}H]MTX found in the cells after 1 or 24 hr remained constant at 0.08–0.10 μg/10^8 cells. With [^{3}H]MTX-MSA-treated mice the level was somewhat higher (0.15 μg/10^8 cells), although the variation of intracellular [^{3}H]MTX content after 24 hr was not statistically significant (see Table 3).

When the ascitic cells of [^{3}H]MTX-, [^{3}H]MTX-BSA-, [^{3}H]MTX-MSA-, and [^{3}H]-MTX-dextran T70-treated mice were lysed and the radioactive content of both lysate and membrane portions was measured, 90% of the tritiated material associated with the cell was found in the cell lysate. Lyophilization and analysis of this extract by chromatography in system A showed that the radioactivity migrated with the MTX marker, indicating that MTX was not metabolized by the cell to other products or, after this treatment, bound to dihydrofolate reductase.

In order to determine whether the albumin portion of MTX-BSA migrated into the cell with the MTX, [^{125}I]MTX-BSA was injected intraperitoneally into mice 6 days after inoculation with 10^6 L1210 cells. After 1 hr approximately 50% of the injected radioactivity was found in the extracellular ascitic fluid; 0.08% was found in the cells. After 24 hr, these values had dropped to 6% and 0.02%, respectively. When both the cell membrane and the cell lysate portions of the 24-hr sample were monitored for radioactivity, 80% of the radioactivity was associated with the cell membrane. The very low levels of activity found in the cell lysate probably reflect minor hydrolytic products of the albumin, such as iodotyrosine, rather than a few large protein fragments carried in with MTX.

DISCUSSION

MTX has been coupled to BSA, MSA, and several high molecular weight dextrans by condensing a terminal carboxyl group of the antifolate molecule, in carbodiimide-promoted reactions, to reactive functions contained in these polymers. ε-Amino groups of lysine residues present in the albumins offered suitable points of attachment to the proteins, and reactive pri-

mary amino substituents introduced into the dextrans, according to procedures previously outlined by Mell *et al.* (5), provided the necessary linkage sites for the carbohydrates. The reactions proceeded efficiently in aqueous bicarbonate-buffered solutions at pH 7.6, and the products were isolated by chromatography on Bio-Gel P-100. They showed absorbance spectra similar to free MTX, were essentially homogeneous on polyacrylamide gel electrophoresis, and were freely soluble in aqueous media. Using the known extinction coefficient of MTX at 370 nm (11), an average uptake of 80 mg/g of high molecular weight carrier could be calculated. This corresponds approximately to 12 moles of MTX per mole of albumin.

Measurement of the free and bound MTX levels developed in BDF_1 mice treated with a single dose of either free MTX or MTX covalently bound to these high molecular weight carriers indicated that the animals treated with the latter complexes demonstrated a higher, more prolonged extracellular concentration of the drug (MTX bound to carrier) than animals treated with the free agent (Fig. 1 and Table 3). In addition, administration of MTX covalently bound to BSA was as effective as free MTX when used in treatment of the ascitic L1210 leukemia carried by BDF_1 mice (Table 2). This ability of the albumin complex to maintain an elevated potential supply of MTX, both in the serum and within the area of injection, suggests that when administered directly into a solid tumor or body cavity containing such a malignancy the derivative may again remain localized and thus be therapeutically beneficial. Treatment directly with free MTX is presently known to be ineffective in such a situation because of its rapid clearance and excretion (16). Surprisingly, the MTX-dextran complexes, which comprised a wide range of molecular weights, were unsuccessful in prolonging the survival time of BDF_1 mice carrying the L1210 tumor, although they did produce significant levels of MTX within the L1210 cells (Table 3).

The amount of radioactivity found in L1210 cells 1 and 24 hr after injection of leukemic mice with tritiated MTX-BSA or MTX–dextran T70 remained fairly constant, at about 0.1 $\mu g/10^8$ cells (Table 3). In contrast, injection of noncovalently bound, labeled MTX led to a rapid fall in intracellular MTX levels, from an initial 0.20 $\mu g/10^8$ cells at 1 hr to 0.075 $\mu g/10^8$ cells at 24 hr. MTX-MSA, which also led to high initial levels of [^{3}H]MTX (0.22 $\mu g/10^8$ cells), again showed a decrease after 24 hr. A similar rapid efflux of free MTX from L1210 cells *in vivo* between 1 hr (maximum concentration) and 20 hr was reported by Sirotnak and Donsbach (17).

Binding of MTX to the host's own serum, MSA, was used to eliminate possible immunological reactions to the dextran and BSA derivatives of MTX. In general, however, the MSA and BSA complexes proved to be equally effective (Fig. 1), although a rather narrow threshold for toxicity was indicated with the MSA derivative. This observation, together with the elevated intracellular levels of MTX recorded after 24 hr, suggested that the host albumin is particularly effective in carrying the drug to, and possibly in facilitating entry through, the cell membrane. The basis of the toxic effects noted with the higher dosages of the MTX derivatives is not yet known, but may become evident when drug distribution after therapy has been carefully evaluated.

It is of interest that the intracellular levels of MTX (1.66×10^{-4} $\mu mole/10^8$ cells) *in vivo* measured after 24 hr by the technique described in this paper correspond well to the previously recorded levels of dihydrofolate reductase in L1210 cells [1–2 $\times 10^{-4}$ $\mu mole/10^8$ cells (18, 19)].

The MTX derivatives used in these experiments changed the mobility and pharmacological properties of the parent drug. Their ability to remain in a specific locality as a potential source of free MTX, together with their altered molecular structure, suggests their use both in the treatment of solid tumors and as antagonists for malignancies occurring in isolated body cavities.

ACKNOWLEDGMENTS

The authors thank Dr. Frank M. Huennekens and Ms. Karin S. Vitols for their critical reviews of this manuscript.

REFERENCES

1. Cosulich, D. B., Seeger, D. R., Fahrenbach, M. J., Roth, B., Mowat, J. H., Smith, J. M., Jr. & Hultquist, M. E. (1951) *J. Am. Chem. Soc.*, 73, 2554–2557.
2. Elliott, R. D., Temple, C., Jr. & Montgomery, J. A. (1968) *J. Org. Chem.*, 33, 533–536.
3. Friedkin, M., Crawford, E. J. & Plante, L. T. (1971) *Ann. N. Y. Acad. Sci.*, 186, 209–213.
4. Johns, D. G., Farquhar, D., Wolpert, M. K., Chabner, B. A. & Loo, T. L. (1973) *Drug Metab. Disp.* 1, 580–589.
5. Mell, G. P., Whiteley, J. M. & Huennekens, F. M. (1968) *J. Biol. Chem.*, 243, 6074–6075.
6. Low, L. W., Dunn, T. B., Boyle, P. J. & Miller, J. H. (1949) *J. Natl. Cancer Inst.*, 10, 179–192.
7. Foley, G. E., Drolet, B. P., McCarthy, R. E., Goulet, K. A., Dokos, J. M. & Filler, D. A. (1960) *Cancer Res.*, 20, 930–939.
8. Semenza, G. (1960) *Helv. Chim. Acta*, 43, 1057–1068.
9. Cosulich, D. B. & Smith, J. M., Jr. (1948) *J. Am. Chem. Soc.*, 70, 1922–1926.
10. Sonoda, S. & Schlamowitz, M. (1972) *J. Immunol.*, 108, 807–818.
11. Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr. & Hultquist, M. E. (1949) *J. Am. Chem. Soc.*, 71, 1753–1758.
12. Harding, N. G. L. (1971) *Ann. N. Y. Acad. Sci.*, 186, 270–283.
13. McConahey, P. J. & Dixon, F. J. (1966) *Int. Arch. Allergy* 29, 185–189.
14. Angier, R. B. & Curran, W. V. (1959) *J. Am. Chem. Soc.*, 81, 2814–2818.
15. Geran, R. I., Greenberg, N. H., MacDonald, M. M., Schumacher, A. M. & Abbott, B. J. (1972) *Cancer Chemother. Rep. Part 3*, 3, 7.
16. Anderson, L. L., Collins, G. J., Ojima, Y. & Sullivan, R. D. (1970) *Cancer Res.*, 30, 1344–1348.
17. Sirotnak, F. M. & Donsbach, R. C. (1973) *Cancer Res.*, 33, 1290–1294.
18. Goldman, I. D., Lichtenstein, N. S. & Oliverio, V. T. (1968) *J. Biol. Chem.*, 243, 5007–5017.
19. Rader, J. I., Niethammer, D. & Huennekens, F. M. (1974) *Biochem. Pharmacol.*, 23, 2057–2059.